The Effect of Embryological Development on Phosphatidylethanolamine Methyltransferase, Phosphatidyldimethylethanolamine Methyltransferase and Choline Phosphotransferase of Rabbit Liver Microsomes

W.E. CORNATZER*, DENNIS R. HOFFMAN and **JUDY A. HANING**, *Department of Biochemistry, Ireland Research Laboratory, University of North Dakota, Grand Forks, ND 58202*

ABSTRACT

The effect of embryological development on the two biosynthetic enzymes involved in phosphatidylcholine biosynthesis in liver microsomes of -12, -9, 0, +4, +14, +36 day old rabbits has been determined. The specific activity (pmol phosphatidylcholine formed/min/mg microsomal protein) of the phosphatidyletanolamine methyltransferase in the liver microsomes is very low before birth and a 33% increase at birth occurs when compared to the -12 day old fetal livers. The pmol of phosphatidylcholine formed/min/mg protein by the choline phosphotransferase pathway in fetal liver microsomes is 5, 10, 73, 199, 107 and 307 times greater than by the phosphatidylethanolamine methyltransferase pathway for -12, -9, 0, +4, +14, +36 day old rabbits, respectively. The specific activities of the choline phosphotransferase in the liver microsomes increased from the -12 day old fetal livers to 1.6, 19, 73, 39, 27 times for the -9, 0, +4, +14 and +36 day old animals, respectively. The choline phosphotransferase pathway in comparison to the phosphatidylethanolamine methyltransferase pathway is providing the major phosphatidylcholines in the membranes of the endoplasmic reticulum before birth and early fetal development of the rabbit.
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INTRODUCTION

Phosphatidylcholine (PC) is the major phospholipid in plasma, nuclear, mitochondrial and endoplasmic reticular membranes of all cells. Phosphatidylcholine represents 45% of total lipid phosphorus of mitochondria, 48.5% of total lipid phosphorus of microsomes (1,2) and 37.4% of total lipid phosphorus of plasma membrane (3).

Phosphatidylcholine biosynthesis in endoplasmic reticulum is known to occur by two major different pathways (4,5). These two pathways are under different biological controls (6-9) and provide different phosphatidylcholines with different fatty acids (10). The Kennedy pathway (4) involves the enzyme choline phosphotransferase, which catalyzes the following reaction: cytidine diphosphocholine + 1,2-diacylglycerol to form PC + CMP. The Bremer-Greenberg (5) pathways involve phosphatidylethanolamine methyltransferase, which catalyzes the following reaction: phosphatidylethanolamine (PE) plus three S-adenosylmethionines with progressive methylation of PE to form PC and three S-adenosylhomocysteines.

During growth and development, biochemical changes have been observed in endoplasmic reticulum (11) and enzyme systems (12-14). Miller and Cornatzer (15) have shown a progressive increase in concentration (μg phospholipid phosphorus/mg protein) of microsomal phosphatidylcholine during development in the embryos of rabbits at -12, -9, 0, +2, +9 and +14 days of age. Chepenik et al. (16) have shown that the rat embryo is able to synthesize PC by the choline phosphotransferase.

A study of the enzymatic activity of the two enzymes, choline phosphotransferase and phosphatidylethanolamine methyltransferase involved in PC biosynthesis in liver microsomes of fetal and newborn rabbits (-12, -9, 0, +4, +14, +36 days old) has been made.

MATERIALS AND METHODS

Animals

New Zealand white rabbits were maintained on Purina rabbit chow checkers and water. The animals were housed in steel wire-bottom cages and were allowed free access to food and water. After the desired gestation interval, the pregnant female rabbit was killed by stunning and bleeding, and the fetal rabbits were removed as soon as possible. Livers were pre-

*To whom correspondence should be addressed.

pared from fetal and young rabbits at -12, -9, 0, +2, +9, +14 and +36 days of age.

Microsomal Preparation

The removed livers were rinsed with cold water, blotted and homogenized in ice-cold 1 mM $MgCl_2$-0.25M sucrose in a Potter-Elvehjem homogenizer with a Teflon pestle. The nuclear and mitochondrial fraction were separated from the homogenate by centrifuging for 10 min at 14,500 g. The supernatant solution was centrifuged at 78,450 g for 60 min to sediment the microsomal pellet. This microsomal preparation as shown by enzyme assay gives very little mitochondria contamination (15). Protein was determined by a modified Biuret method (17) and the enzymes involved in PC biosynthesis assayed.

Phosphatidylethanolamine Methyltransferase Assay

The methylation pathway was measured by a modified method of Bremer and Greenberg (5). The reaction mixture contained 2 mg egg phosphatidylcholine (Sigma Chemical Co., St. Louis, MO), 0.9 mM sodium deoxycholate, 0.3 M Tris-HCl buffer (pH 8.6), 0.2 mM S-adenosyl-L-[methyl-^{14}C]methionine (0.1 mCi/mmol, New England Nuclear, Boston, MA) and microsomes (1-2 mg protein) in a final volume of 1.4 ml. The assay was initiated by adding microsomes, containing endogenous PE, reaction time was 15 min at 37 C, and the reaction was terminated with 0.5 ml of concentrated HCl. The ^{14}C-labeled reaction product, phosphatidylcholine, was extracted according to the Bligh and Dyer procedures (18,19). Radioactivity was measured in a Packard liquid scintillation spectrometer. In this mixture, the egg phosphatidylcholine acted as an activator (19).

Phosphatidyldimethylethanolamine Methyltransferase Assay

The method of Rehbinder and Greenberg (20) was used to determine the activity of phosphatidyldimethylethanolamine methyltransferase which catalyzes the last step in the methylation of PE to PC. The reaction mixture contained 3.0 mM egg phosphatidyldimethylethanolamine as substrate (Avanti Polar Lipids, Birmingham, AL), 6.3 mM sodium deoxycholate, 0.3M Tris-HCl buffer (pH 8.6), 0.35 mM S-asenosyl-L-[methyl-^{14}C]methionine (0.1 mCi/mmol, New England Nuclear, Boston, MA), and microsomes (1-2 mg) in a final volume of 1.15 ml. Reaction time was 15 min at 37 C. The reaction was initiated by adding the microsomes, and was terminated by adding 0.1 ml of concentrated HCl. The ^{14}C-labeled phosphatidylcholine was isolated with methanol/chloroform (18,19) and radioactivity determined.

Choline Phosphotransferase Assay

The method of Kennedy and Weiss (4) was used to determine the activity of CDP-choline:1,2-diacylglycerol choline phosphotransferase (choline phosphotransferase). The reaction mixture contained 2 μmol, 1,2-diacylglycerol emulsified in 0.1 ml of 1% Tween-20 (Sigma Co., St. Louis, MO), 10 μmol $MgCl_2$, 0.5 μmol cytidine diphosphate 1,2-^{14}C-choline (sp act 4 $\times$ 10^5 cpm/μmol, ICN Tracerlab, Irvin, CA), and microsomes (0.5-2.0 mg protein) in a final volume of 1.3 ml. The diacylglycerol was prepared from egg lecithin by the method of Gurr et al. (21), and purified by the chromatographic method of Barron and Hanahan (22). Reaction time was 6 min. The reaction was initiated by adding the microsomes, and was terminated by adding 3.0 ml of 95% ethanol. The ^{14}C-labeled phosphatidylcholine was isolated by repeated extraction with ethanol and chloroform (4).

RESULTS AND DISCUSSION

The enzymatic activities of phosphatidylethanolamine methyltransferase, phosphatidyldimethylethanolamine methyltransferase and choline phosphotransferase were linear with time and concentration of microsomal enzyme. The data have been presented previously (23).

The effects of embryological development on the two biosynthetic enzymes involved in PC biosynthesis in liver microsomes is given in Table 1. At birth there was over a 5-fold increase in liver weight and over an 8-fold increase in microsomal protein when compared to the -9 day fetal livers. The percentage increase in the specific activity (pmol phosphatidylcholine formed/min/mg protein) of phosphatidylethanolamine methyltransferase in fetal and young rabbit microsomes over -12 days old compared to -9, 0, +4, +14 and +36 days old was -16, 33, 86, 86, -56, respectively. The specific activity of the phosphatidylethanolamine methyltransferase in the liver microsomes is very low before birth and a 33% increase occurs at birth.

A number of other enzymes have low activity in fetal liver and a rapid increase at birth occurs: glucose-6-phosphatase (24), carbamyl phosphate synthetase (25), superoxide dismutase (26), gamma glutamyltranspetidase (27), isocitrate dehydrogenase, aspartate aminotransferase, malate dehydrogenase (28), bran-

TABLE 1

Phosphatidylcholine Biosynthesis During Embryological Development in Rabbit Liver Microsomes

Age	No. of Animals	Liver wt	Microsomal protein	Specific activity (phosphatidylcholine formed/min/mg protein)		
				Phosphatidyl-ethanolamine methyltransferase	Phosphatidyl-dimethyl-ethanolamine methyltransferase	Choline phospho-transferase
		(g)	(mg/whole liver)	(pmol)		
-12	40	0.07 ±0.03	4.38 ±0.88	49.7 ± 1.2 (299 ± 42)	238.0 ± 32.5 (1046.0 ± 142.5)	258 ± 42 (1132 ± 180)
-9	24	0.514 ± 0.18	9.91 ±0.65	41.8 ± 1.4 661 ± 402)	614.6 ± 2.65 (70,680 ± 309)	421 ± 10 (50,000 ± 501)
0	10	2.69 ±0.88	80.0 ±14.5	66.1 ± 6.5 (10,145 ± 820)	1224.9 ± 78.8 (188,070 ± 9680)	4780 ± 130 (734,600 ± 42,400)
+4	5	3.21 ±0.33	67.6 ±7.82	92.3 ± 18.7 (13,048 ± 2620)	2351.0 ± 315.1 (323,430 ± 44,120)	18,420± 2900 (2,578,800± 409,500)
+14	9	5.97 ±1.89	223.4 ± 65.3	92.3 ± 4.7 (16,796 ± 4094)	1886.6 ± 294.1 (405,739 ± 5537)	9900 ±2300 (2,217,200± 553,300)
+36	3	40.2 ± 8.2	1856.2 ± 327.7	22.07 ± 68 (40,800 ± 5860)	447.1 ± 36.1 (822,500 ± 73,800)	6750 ±1090 (12,762,200 ± 420,710)

Values (followed by standard deviations) are the means of duplicate determinations of specific activity. The values in parentheses are total liver microsomal activity (pmol phosphatidylcholine formed/min/total liver microsomes). All animals were New Zealand white rabbits. Pregnant females with predetermined gestation periods were sacrificed, fetal rabbits removed and livers pooled for preparation of microsomes. Livers at age 4 days and older were used individually and not pooled.

ched chain 2-oxo acid dehydrogenase (29).

There is a progressive increase in the specific activity of phosphatidyldimethylethanolamine methyltransferase during development of the fetal livers. This assay measures the last methylation of PE to PC. The first methylation of PE to PC is rate-limiting (9). The fetal liver microsomes have the ability to methylate the last step in the conversion of PE to PC if external substrate is provided as in the phosphatidyldimethylethanolamine methyltransferase assay. The low activity of the phosphatidylethanolamine methyltransferase in fetal livers before birth may be due to a low concentration of an activator such as a hormone or the concentration of an inhibitor (8). Thyroxine increases the activity of a number of fetal enzymes, arginase (30), aspartate aminotransferase (31), NADPH-cytochrome c dehydrogenase (32) and glucose-6-phosphatase (24). The administration of thyroxine resulted in a significant increase in the specific activity of phosphatidylethanolamine methyltransferase in the liver microsomes of hyperthyroid rats and a reduction of the specific activity occurred in hypothyroid states (33).

The pmol of phosphatidylcholine formed/min/mg protein by the choline phosphotransferase pathway in the -12 day fetal liver microsomes is 5 times greater than by the phosphatidylethanolamine methyltransferase pathway. This value is 10 and 72 times at -9 and 0 day old fetal livers, respectively, for the choline phosphotransferase. The specific activities of the choline phosphotransferase in the liver microsomes increases from the -12 day old fetal liver to 1.6, 19, 73, 39, 27 times for the -9, 0, +4, +14 and +36 day old animals, respectively. Thus, the choline phosphotransferase pathway in comparison to the phosphatidylethanolamine methyltransferase pathway is providing the major phosphatidylcholines in the membranes of the endoplasmic reticulum before birth and early fetal development of the rabbit.

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REFERENCES

1. Johnson, J.D., and Cornatzer, W.E. (1969) Proc. Soc. Exp. Biol. Med. 131, 474-478.
2. Tsao, S.S., and Cornatzer, W.E. (1967) Lipids 2, 41-46.
3. Pfleger, R.C., Anderson, H.G., and Snyder, F. (1968) Biochemistry 7, 2826-2833.
4. Kennedy, E.P., and Weiss, S.B. (1956) J. Biol. Chem. 222, 193-213.
5. Bremer, J., and Greenberg, D.M. (1960) Biochim. Biophys. Acta 37, 173-175.
6. Hoffman, D.R., Uthus, E.O., and Cornatzer, W.E. (1980) Lipids 15, 439-446.
7. Hoffman, D.R., Haning, J.A., and Cornatzer, W.E. (1981) Can. J. Biochem. 59, 543-550.
8. Hoffman, D.R., Haning, J.A., and Cornatzer, W.E. (1981) Lipids 16, 561-567.
9. Hoffman, D.R., Haning, J.A., and Cornatzer, W.E. (1981) Int. J. Biochem. 13, 745-748.
10. Rytter, D., Miller, J.E., and Cornatzer, W.E. (1968) Biochim. Biophys. Acta 152, 418-421.
11. Lathe, G.H., and Ricketts, T.R. (1964) Q. Exp. Physiol. 49, 74-80.
12. Dallner, G., Siekewitz, P., and Palade, G.E. (1965) Biochem. Biophys. Res. Commun. 20, 135-141.
13. Lint, M.F., Lathe, G.H., Ricketts, T.R., and Silman, G. (1964) Q. J. Exp. Physiol. 49, 66-73.
14. Nemeth, A.M. (1954) J. Biol. Chem. 208, 773-776.
15. Miller, J.E., and Cornatzer, W.E. (1966) Biochem. Biophys. Acta 125, 534-541.
16. Chepenik, K.P., Borst, D.E., and Waite, B.M. (1977) Dev. Biol. 60, 463-472.
17. Lee, Y.P., and Lardy, H.A. (1965) J. Biol. Chem. 240, 1427-1436.
18. Bligh, E.G., and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911-917.
19. Hoffman, D.R., and Cornatzer, W.E. (1981) Lipids 16, 533-540.
20. Rehbinder, D., and Greenberg, D.M. (1965) Arch. Biochem. Biophys. 109, 110-115.
21. Gurr, M.I., Brindley, D.N., and Hübscher, G. (1965) Biochim. Biophys. Acta 98, 486-501.
22. Barron, E.J., and Hanahan, D.J. (1958) J. Biol. Chem. 231, 493-503.
23. Skurdal, D.N., and Cornatzer, W.E. (1975) Int. J. Biochem. 6, 579-583.
24. Greengard, O. (1969) Science 163, 891-895.
25. Raymond, Y., and Shore, G.S. (1981) Biochim. Biophys. Acta 656, 111-119.
26. Mavelli, I., Autuori, F., Diori, L., Spinedi, A., Ciriolo, M.R., and Rotilio, G. (1981) Biochem. Biophys. Res. Commun. 102, 911-916.
27. Igavashi, T., Satoh, T., Ueno, K., and Kitagawa (1981) Life Sci. 29, 483-491.
28. Felder, M.R., Church, W.K., and Shaffer, J.B. (1982) Oncology 39, 382-390.
29. May, E.E., May, M.E., Aftring, R.P., and Buse, M.G. (1982) Biochem. J. 204, 487-492.
30. Greengard, O. (1971) Essays Biochem. 7, 159-205.
31. Herzfeld, A., and Greengard, O. (1971) Biochim. Biophys. Acta 237, 88-98.
32. Greengard, O., and Dewy, H.K. (1968) J. Biol. Chem. 243, 2745-2749.
33. Cornatzer, W.E., Hoffman, D.R., and Haning, J.A. (1982) Fed. Proc. 41, 543.

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Effect of Dietary Animal and Vegetable Protein on Gallstone Formation and Biliary Constituents in the Hamster[1]

SUSAN MAHFOUZ-CERCONE[a], JAMES E. JOHNSON[b] and GEORGE U. LIEPA[a,*],
[a]Department of Nutrition and Food Sciences and [b]Department of Chemistry, Texas Woman's University, PO Box 24134, Denton, TX 76204

ABSTRACT

A gallstone-inducing diet was utilized to examine the effect of dietary proteins (casein, soybean and cottonseed) on gallstone formation. Casein produced gallstones in 100% of the animals; however, soybean or cottonseed proteins reduced gallstone incidence to 32% and 0%, respectively. In an effort to ascertain the mechanisms responsible for gallstone formation, serum cholesterol and the 3 primary biliary constituents (bile acids, phospholipids and cholesterol) were measured. Casein produced a 4-fold increase in biliary cholesterol, whereas soybean and cottonseed yielded a 3- and 2-fold increase, respectively, when compared to a commercial diet (Purina, no. 5001). Serum cholesterol was reduced by substituting dietary vegetable protein for animal protein. This study suggests that substitution of vegetable for animal protein in the diet can decrease gallstone formation in hamsters. It is proposed that this reduced gallstone formation is due to the decreased concentrations of biliary cholesterol induced by dietary modification.
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INTRODUCTION

Clinical and epidemiological data reveal that diet plays a significant role in the development of gallstone disease. In 1952, Dam and Christensen (1) reported that cholesterol gallstones could be produced in hamsters by diet alone. The diet, referred to as the Dam diet, contained casein as a protein source, sucrose as the primary source of carbohydrate, and no fat. Gallstones were produced in 80-100% of the hamsters within 6-8 weeks.

To date, most of the research emphasizing the effects of diet on gallstone formation has concentrated on dietary cholesterol (2-4), fat (5,6), carbohydrate (1,7,8) and excessive caloric intake (9,10). Insufficient attention has been devoted to dietary proteins. This is surprising, since dietary proteins may effect biliary cholesterol concentrations and seem to influence serum lipids, especially cholesterol (12-14).

In 1958, Olson et al. (11) observed a decrease in serum cholesterol concentrations in human subjects when their daily protein intake was changed from 100 g of animal protein to 25 g of vegetable protein. Kritchevsky et al. (12) and Park and Liepa (13) have also observed hypercholesterolemic effects of dietary animal protein when compared with dietary vegetable protein in experimental animals. Sirtori et al. (14) studied the effect of dietary protein on serum lipids using hypercholesterolemic male subjects. The substitution of vegetable proteins for animal proteins produced a 32% decrease in serum cholesterol concentration. Additional studies have also indicated that dietary vegetable proteins when compared to animal proteins are hypocholesterolemic (15-18).

Recognizing that dietary protein can influence serum lipids, Kritchevsky and Klurfeld (19) recently demonstrated that dietary vegetable protein markedly reduced the formation of cholesterol gallstones in hamsters. In their study, 58% of the hamsters developed gallstones when their diet contained casein, as compared to 14% of the hamsters which were fed a diet containing soybean protein. Furthermore, once cholesterol gallstones were present in the hamster, significant dissolution of stones was accomplished with administration of a soybean protein diet.

There is clear evidence that diet has an effect on cholelithiasis, although the mechanisms are not clearly defined. The relationship between dietary protein and cholelithiasis is one of particular interest, since protein derived from animal sources has a greater tendency to elevate plasma cholesterol concentration than protein derived from plant sources. The purpose of this study was to examine the effect of dietary proteins (casein, soybean and cottonseed) on cholesterol gallstone formation in the hamster. In an effort to ascertain the mechanism responsible for gallstone formation, the 3 primary

[1]Preliminary reports of portions of this work were presented at the XIIth International Congress of Nutrition and at the 73rd annual AOCS meeting. Liepa, G., and Mahfouz, S. (1982) J. Am. Oil Chem. Soc. 59, 316A.

*To whom correspondence should be addressed.

TABLE 1

Experimental Diets[a,b]

Group	Composition (%)
I[c]	74.3 Carbohydrate (sucrose)
	20.0 Protein (casein)[d]
	5.0 Mineral mix[e]
	0.5 Vitamin mix[f]
	0.2 Choline chloride
II	74.3 Carbohydrate (sucrose)
	20.0 Protein (soybean)[g]
	5.0 Mineral mix
	0.5 Vitamin mix
	0.2 Choline chloride
III	74.3 Carbohydrate (87% sucrose, 13% from cottonseed flour)[h]
	20.0 Protein (cottonseed)
	5.0 Mineral mix
	0.5 Vitamin mix
	0.2 Choline chloride
IV	Control – Purina Rodent Chow 5001

[a] All diets were obtained from the United States Biochemical Corporation, Cleveland, OH. Cottonseed protein was supplied by Texas A&M University.

[b] All diets are isocaloric providing ca. 3.5 kcal/g of diet.

[c] Dam diet.

[d] Obtained from casein isolate consisting of 85% protein, 1.8% carbohydrate, 1.0% fat, 0.1% fiber, 1.8% ash and 9.8% moisture.

[e] Mineral mixture (%): 0.009 $Al_2(SO_4)_3 \cdot 24\ H_2O$; 11.280 $CaHPO_4 \cdot 2\ H_2O$; 6.860 $CaCO_3$; 30.830 $Ca(C_6H_5O_7)_2 \cdot 4\ H_2O$; 0.008 $CuSO_4$; 1.526 Fe $(NH_4)(C_6H_5O_7)_2$; 3.520 $MgCO_3$; 3.830 $MgSO_4$; 0.020 $MnSO_4$; 12.470 KCl; 0.004 KI; 21.880 KH_2PO_4; 7.710 NaCl; 0.050 NaF; 2×10^{-6} Cr $(C_2H_3O_2)_3$; 5×10^{-5} $ZnCO_3$.

[f] Vitamin mixture (g/kg): 0.1100 *p*-aminobenzoic acid; 1.0170 vitamin C; 0.0004 biotin; 0.0660 Ca pantothenate; 3.7150 choline citrate; 0.0020 folic acid; 0.1100 inositol; 0.0500 vitamin K; 0.0090 nicotinic acid; 0.0220 pyridoxine-HCl; 0.0220 riboflavin; 0.0020 thiamin-HCl; 0.0390 vitamin A (500,000 U/g); 0.0290 vitamin B_{12}; 0.0040 vitamin D_2 (500,000 U/g); 0.4850 vitamin E acetate (250 U/g).

[g] Obtained from soy protein isolate consisting of 86.0% protein, 3.8% carbohydrate, 0.8% fat, 0.1% fiber, 3.8% ash and 5.5% moisture.

[h] Obtained from cottonseed flour consisting of 56.6% protein, 25.0% carbohydrate, 3.4% fat, 2.6% fiber, 6.9% ash and 5.5% moisture. Free gossypol levels were at 0.02% (200,000 ppm).

biliary constituents (bile acids, phospholipid and cholesterol) and serum cholesterol were quantitatively measured.

METHODS

Description and Care of Animals

Sixty-nine male hamsters *(Mesocricetus auratus)* weighing 60 ± 5 g were obtained from Engle Laboratories (Farmersburg, IN). The animals were individually housed in a well ventilated room which was artificially illuminated from 6 a.m. to 6 p.m. Food consumption and body weight for each animal were recorded 4 times per week. All animals were maintained in accordance with the guidelines stated by the National Research Council.

On arrival, the animals were fed Purina Rodent Chow 5001, ad libitum for 7 days. After this initial equilibration period, the animals were randomly assigned to 1 of 3 experimental groups or to a control group and were fed the experimental diets (Table 1) for 45 days. Diets and water were provided ad libitum throughout the experiment.

Collection and Preparation of Samples for Analysis

Animals were killed after having been fasted for ca. 12 hr. Between 9 and 11 a.m., the hamsters were anesthetized with ether, after which time bile was aspirated directly from the gallbladder with a 10-μl Hamilton syringe. The gallbladder was excised and examined for the presence of gallstones. The blood samples were centrifuged for 20 min (4,000 rpm at 4 C) and the plasma was removed. Bile and plasma samples were frozen immediately and stored at −20 C for subsequent chemical analysis.

Analytical Techniques

The 3 major constituents of the gallbladder bile (bile acids, phospholipid and cholesterol) were analyzed quantitatively by the methods of Turley and Dietschy (20), Trudinger (21), and Reyes and Kern (22), respectively. Total serum cholesterol concentration was determined quantitatively using the enzymatic method of Allain et al. (23). The lithogenic index of bile was calculated using methods described by Thomas and Hofmann (24).

Statistical Analysis

The data were analyzed using single factor analysis of variance with 4 groups. Individual comparisons were evaluated using the t-test for independent contrasts and the Newman-Keuls multiple range procedure (25). Values of $p < 0.05$ were considered to be statistically significant.

RESULTS AND DISCUSSION

Effect of Dietary Protein on Animal Growth and on Cholesterol Gallstone Incidence

The rate of growth was similar for all dietary groups (final weights: casein, 105.6 ± 7.2; soybean, 113.5 ± 7.2; cottonseed protein, 106.5 ± 8.6; control, 116.3 ± 5.9); however, gallstone formation was altered significantly. Significantly fewer gallstones were observed when the

TABLE 2

Effect of Dietary Protein on Gallstone Formation and Biliary Constituents of Hamsters

Protein source	n	Gallstones (%)	Absolute concentration (μmol/mol)		
			Bile acid	Phospholipid	Cholesterol
Casein	9	100	140.0 ± 15.4[a,b,A]	17.4 ± 2.3[A]	6.30 ± 0.5[A]
			(85.2 ± 0.7)[c,1]	(10.5 ± 0.4)[1]	(4.3 ± 0.7)[1]
Soybean	18	31.6	140.9 ± 7.3[A]	17.4 ± 1.4[A]	5.18 ± 0.03[A]
			(86.4 ± 0.4)[1]	(10.4 ± 0.5)[1]	(3.2 ± 0.1)[1]
Cottonseed	15	0.0	116.9 ± 7.6[A]	12.0 ± 1.3[B]	3.03 ± 0.2[B]
			(88.9 ± 0.5)[2]	(8.8 ± 0.5)[2]	(2.3 ± 0.1)[2]
Control	17	0.0	223.8 ± 11.7[B]	16.7 ± 1.3[A]	2.74 ± 0.2[B]
			(92.1 ± 0.3)[3]	(6.8 ± 0.3)[3]	(1.1 ± 0.1)[3]

[a]$\bar{X}$ ± SEM.
[b]Values without common superscripts are different at $P < 0.05$. Capital letters are used to denote differences in absolute concentrations and numbers denote differences in relative concentrations.
[c]Relative concentration (molar %).

source of dietary protein was a vegetable product (soybean or cottonseed) as compared to an animal product (casein) (Table 2). These results are in agreement with the observations reported by Kritchevsky and Klurfeld (19), who found dietary animal protein (casein) to be more lithogenic than vegetable protein (soybean).

The present study indicates that cottonseed protein may be even more effective at decreasing the incidence of gallstones than soybean protein; however, the cottonseed concentrate which was used to prepare the experimental diet was not as pure as the soy protein and casein and consequently it is possible that other factors in the concentrate altered the rate of gallstone formation. No gallstones were found in the gallbladder or biliary duct of the control animals.

Effect of Dietary Protein on Biliary Constituents

In an effort to ascertain the mechanism or mechanisms responsible for gallstone formation with respect to the experimental diets, the bile was quantitatively analyzed for the 3 major biliary constituents (bile acids, phospholipid and cholesterol) (Table 2).

No significant differences were observed between the absolute concentrations of bile acids in the experimental groups (Table 2). However, the mean absolute concentration of bile acids was significantly higher in the control group than in any experimental group ($p < .05$). The relative concentration of bile acids in gallbladder bile of animals fed casein or soybean as the protein source did not differ significantly; however, the percentage of bile acids in hamsters fed cottonseed protein was significantly greater than with either of the other proteins. The control group had a significantly higher relative concentration of bile acids than any of the experimental groups.

Bile acid concentration has been shown to play a role in gallstone development. American Indian women suffering from an increased incidence of gallstone disease tend to have decreased rates of hepatic bile acid secretion, a decreased bile acid pool size, and an increased secretion of hepatic cholesterol (26). Subjects who have had either an ileal resection or an intestinal bypass also are predisposed to gallstone disease. The predisposition is thought to be due to an inadequate reabsorption of bile acid and a concomitant lowering of the bile acid pool (27,28).

Although studies carried out on gallstone patients (29,30) show no change in the relative concentrations of biliary phospholipids, recent work with hamsters (31) indicates that the formation of lithogenic bile was due to a relative decrease in biliary phospholipid concentration.

The absolute concentration of phospholipid present in bile taken from hamsters fed cottonseed protein was significantly lower than that from animals fed casein, soybean protein or laboratory chow, but no differences were noted among the latter 3 groups (Table 2). The relative concentration of biliary phospholipid of animals fed cottonseed protein was significantly lower than that of animals fed casein or soybean protein with no difference between the latter 2 groups. The relative concentration of biliary phospholipid was significantly lower in the control animals when compared to any experimental group.

When casein was the protein source, hamsters exhibited a higher absolute and relative (molar %) concentration of biliary cholesterol

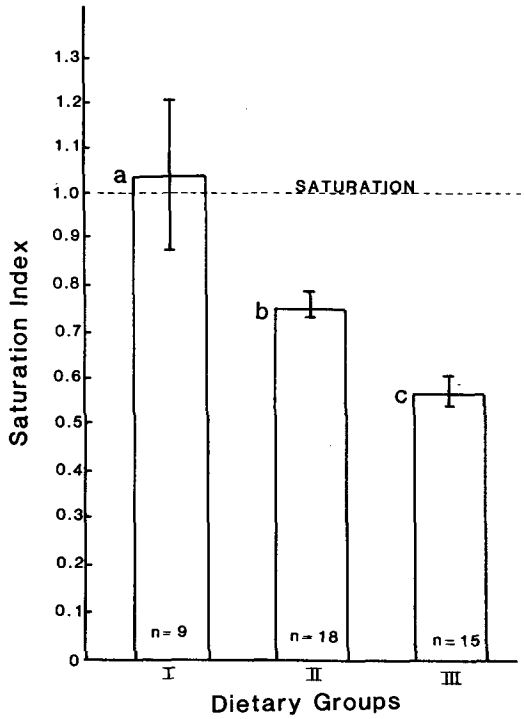

FIG. 1. Lithogenic index of bile taken from hamsters fed a variety of dietary proteins. Values are $\bar{X}$ ± SEM; values without common superscripts are different at $P < 0.05$. Dietary groups: diet I (Dam diet), diet II (soybean used as a protein source), diet III (cottonseed used as a protein source).

than did animals fed vegetable proteins or the control diet (Table 2). Casein-fed animals had a higher percentage of biliary cholesterol than animals fed soybean protein; however, this difference was not statistically significant. Animals fed cottonseed protein had a significantly lower percentage of biliary cholesterol when compared to either casein- or soybean protein-fed animals. The percentage of biliary cholesterol was significantly lower in the control group when compared to any of the experimental groups. Kajiyama (31) recently reported similar values for biliary cholesterol (molar %) when using the Dam diet with casein as a protein source.

According to Redinger and Small (32), if the biliary cholesterol concentration comprises greater than 5% of the total composition of bile, cholesterol will precipitate out of solution, possibly resulting in the formation of cholesterol gallstones. Also, when the concentration of bile acids is below 80%, gallstone formation will begin. It is suggested that the increase in biliary cholesterol, and the concomitant decrease in bile acids, were responsible for gallstone formation in this study.

Saturation Index

The lithogenic index of saturation was used to evaluate all 3 biliary constituents: bile acids, phospholipids and cholesterol. A saturation index greater than 1.0 suggests a supersaturated bile which promotes possible gallstone formation (30,33). Figure 1 illustrates that casein-fed animals had a saturation index greater than 1.0, which is consistent with the observation that 100% of these animals had gallstones. The soybean and cottonseed proteins produced a significantly lower saturation index when compared to casein, and the animals had a greatly reduced incidence of gallstone formation.

Effect of Dietary Protein on Serum Cholesterol

Serum cholesterol concentration was lowered when casein was replaced with soybean; however, this difference was not statistically significant (Table 3). Dietary cottonseed protein was associated with a significantly lowered total serum cholesterol concentration when compared to other experimental diets. All of the experimental diets exhibited significantly higher total serum cholesterol concentration than did the animals fed laboratory chow.

The preceding finding is not surprising, since a variety of studies (13,14,34-36) have shown that serum cholesterol concentration is de-

TABLE 3

Effect of Dietary Protein on Cholesterol Concentration of Gallbladder Bile and Serum

Protein	Gallbladder bile cholesterol (μmol/ml)	Serum cholesterol (mg/dl)
Casein	$6.30 \pm 0.52^{a,b,A}$ (n=9)	212.60 ± 10.81^{A} (n=16)
Soybean	5.18 ± 0.35^{A} (n=18)	198.24 ± 5.08^{AB} (n=19)
Cottonseed	3.03 ± 0.26^{B} (n=15)	180.61 ± 8.69^{B} (n=16)
Control	2.74 ± 0.26^{B} (n=17)	122.86 ± 7.97^{C} (n=17)

[a] $\bar{X}$ ± SEM.
[b] Values without common capital letter superscripts are different at $P < 0.05$.

creased when animals are fed soy protein in their diets as opposed to casein. Forsythe et al. (34) fed pigs diets containing either soybean meal or casein as well as various concentrations of fats. Results showed that total plasma and HDL cholesterol concentrations were decreased in pigs fed plant protein compared to those fed animal protein. Roy and Schneeman (35) fed mice cholesterolemic diets and found that animals fed soy diets had decreased plasma cholesterol concentration and higher intestinal bile acid concentration than those fed casein diets. Wolfe et al. (36) found similar changes using rabbits, and proposed that the increased excretion of bile acids impaired intestinal cholesterol reabsorption. Kritchevsky (37) and Park and Liepa (13) have proposed that the dietary ratios of arginine and lysine may play a role in the hypocholesterolemic effect of vegetable proteins.

This research indicates a moderate positive correlation ($r = +0.55$) between the concentration of cholesterol in the serum and the concentration of cholesterol in the bile (Table 3). The animals fed casein as the protein source produced the highest concentrations of both serum and biliary cholesterol, as well as the greatest incidence of cholesterol gallstone formation. Cottonseed protein produced significantly decreased concentrations of serum and biliary cholesterol compared to casein-fed hamsters and produced a significant decrease in gallstone incidence.

SUMMARY AND CONCLUSIONS

Total serum cholesterol was quantitatively measured to determine if the type of dietary protein, animal or vegetable, would influence the level of cholesterol in the serum and concomitantly in the bile. This research has supported earlier observations that vegetable proteins are more hypocholesterolemic when compared to animal proteins in both experimental animals and man (11-18) and that there does appear to be a positive correlation between the concentration of cholesterol in the serum and gallbladder bile. Casein-fed animals exhibited the highest mean absolute and relative concentration of biliary cholesterol, and the highest mean serum cholesterol concentration when compared to diets where soybean and cottonseed were the protein sources. Conversely, animals fed cottonseed as the protein source exhibited the lowest absolute concentrations of biliary cholesterol, serum cholesterol and gallstone incidence.

Consideration of the aforementioned information suggests that a reduction in the amount of animal protein in the diet with the substitution of vegetable proteins may offer an alternative treatment for cholesterol cholelithiasis.

More research is needed concerning the comparative effects of dietary protein on cholesterol gallstone formation and biliary lipid composition to determine if all dietary animal proteins are lithogenic when compared to vegetable proteins. Additionally, dietary proteins should be studied to determine if a decrease in biliary saturation with the subsequent dissolution of cholesterol cholelithiasis is possible with dietary intervention.

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REFERENCES

1. Dam, H., and Christensen, F. (1952) Acta Pathol. Microbiol. Scand. 30, 236-42.
2. Chang, S.H., Ho, K.J., and Taylor, C.B. (1973) Arch. Pathol. 96, 417-26.
3. Ho, K.J. (1976) Am. J. Clin. Nutr. 29, 698-704.
4. Christensen, F., Dam, H., and Prange, I. (1952) Acta Physiol. Scand. 27, 315-20.
5. Miettinen, M., Turpeinen, O., Karvonen, M.J., Paavilainen, E., and Elosuo, R. (1976) Ann. Clin. Res. 8, 111-16.
6. Grundy, S.M., and Metzger, A.L. (1972) Gastroenterology 62, 1200-17.
7. Malmros, H. (1969) Lancet 2, 479-84.
8. Christensen, F., Dam, H., and Kristensen, G. (1956) Acta Physiol. Scand. 36, 329-36.
9. Bennion, L.J., and Grundy, S.M. (1975) J. Clin. Invest. 56, 996-1011.
10. Mabee, T.M., Meyer, P., Den Besten, L., and Mason, E.E. (1976) Surgery 79, 460-68.
11. Olson, R.E., Vester, J.W., Gursey, D., Davis, N., and Longman, D. (1958) Am. J. Clin. Nutr. 6, 310-24.
12. Kritchevsky, D., Kolman, R.R., Guttmacher, R.M., and Forbes, M. (1959) Arch. Biochem. Biophys. 85, 444-51.
13. Park, M., and Liepa, G.U. (1982) J. Nutr. 112, 1892-98.
14. Sirtori, C.R., Agradi, E., Conti, F., Mantero, O., and Gatti, E. (1977) Lancet 1, 275-77.
15. Carrol, K.K., and Hamilton, R.M.G. (1975) J. Food Sci. 40, 18-23.
16. Howard, A.N., Gresham, G.A., Jones, D., and Hennings, I.W. (1965) J. Atheroscler. Res. 5, 330-37.
17. Walker, G.R., Morse, E.H., and Overley, V.A. (1960) J. Nutr. 72, 317-21.
18. Hodges, R.E., Lopez, A., Krehl, W.A., and Stone, D.B. (1967) Am. J. Clin. Nutr. 20, 198-208.
19. Kritchevsky, D., and Klurfeld, D.M. (1979) Am. J. Clin. Nutr. 32, 2174-76.
20. Turley, S.D., and Dietschy, J.M. (1978) J. Lipid Res. 19, 924-28.

21. Trudinger, P.A. (1970) Anal. Biochem. 36, 222-25.
22. Reyes, H., and Kern, F. (1979) Gastroenterology 76, 144-50.
23. Allain, C.C., Poon, L.S., Chan, C.S., Richmond, W., and Fu, P.C. (1974) Clin. Chem. 20, 470-75.
24. Thomas, P.J., and Hofmann, A.F. (1970) Gastroenterology 65, 698-700.
25. Winer, B.J. (1971) in Statistical Principles in Experimental Design, McGraw-Hill, New York, NY.
26. Grundy, S.M., Metzger, A.L., and Adler, R.D. (1972) J. Clin. Invest. 51, 3026-30.
27. Grundy, S.M., and Mok, H.Y. (1977) J. Lab. Clin. Med. 89, 354-66.
28. Grundy, S.M. (1979) Eur. J. Clin. Invest. 9, 179-80.
29. Carey, M.C., and Small, D.M. (1978) J. Clin. Invest. 61, 998-1026.
30. Holzbach, R.T., Marsh, M., Olszewski, M., and Holan, K. (1973) J. Clin. Invest. 52, 1467-79.
31. Kajiyama, G., Kubota, S., Sasaki, H., Kawamoto, T., and Miyoshi, A. (1980) Hiroshima J. Med. Sci. 29(3), 133-38.
32. Redinger, R.N., and Small, D.M. (1972) Arch. Intern. Med. 130, 618-30.
33. Hegardt, F.G., Dam, H. (1971) Z. Ernaehrungswiss. 10, 223-33.
34. Forsythe, W.A., Miller, E.R., Hill, G.M., Romsos, D.R., and Simpson, R.C. (1980) J. Nutr. 110, 2467-79.
35. Roy, D.M., and Schneeman, O.B. (1981) J. Nutr. 111, 878-85.
36. Wolfe, B.M., Giovannetti, P., Cheng, D., Roberts, D., and Carrol, K.K. (1981) Nutr. Rep. Intl. 24, 1187-92.
37. Kritchevsky, D. (1979) J. Am. Oil Chem. Soc. 56, 135-39.

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The Absorption and Transport of Dietary Cholesterol in the Presence of Peanut Oil or Randomized Peanut Oil

PATRICK TSO[a]*, GENE PINKSTON[a], DAVID M. KLURFELD[b], and DAVID KRITCHEVSKY[b], [a]*Department of Physiology and Biophysics, University of Tennessee Center for the Health Sciences, Memphis, TN 38163, and* [b]*The Wistar Institute of Anatomy and Biology, Philadelphia, PA 19104*

ABSTRACT

Peanut oil has been shown to be unexpectedly atherogenic for cholesterol-fed rats, rabbits and rhesus monkeys. However, randomization (rearrangement of fatty acids to random distribution) of peanut oil significantly reduced its atherogenicity for rabbits and monkeys. This study was conducted to investigate whether the absorption and transport of dietary cholesterol was altered in the presence of peanut oil or randomized peanut oil, thereby accounting for the difference in the atherogenicity of the two diets. Intestinal lymph fistula rats were infused intraduodenally with a lipid emulsion at a rate of 3 ml/hr. The lipid emulsion contained either peanut oil (control) or randomized peanut oil (experimental) (10 mM), ^{14}C-cholesterol (1.3 mM) and sodium taurocholate (19 mM) in phosphate-buffered saline, pH 6.4. Lymph triglyceride, cholesterol and phospholipid outputs were similar in both groups of rats during fasting and subsequently during lipid infusion. Comparable recovery of ^{14}C-cholesterol from the intestinal lumen and the intestinal mucosa of the control and the experimental rats showed that the absorption and transport of dietary cholesterol were similar in both groups of rats. Analyses of the fatty acid of both lymph and intestinal mucosal lipid again failed to reveal a difference between the 2 groups of rats. It is concluded that the difference in the atherogenicity between the peanut oil and the randomized peanut oil is probably caused by events subsequent to the release of cholesterol containing chylomicrons and very low density lipoproteins by the small intestinal epithelial cells.
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INTRODUCTION

Peanut oil, despite its relatively high iodine value (95 ± 5), is surprisingly atherogenic for cholesterol-fed rats (1), rabbits (2) and rhesus monkeys (3). It is also atherogenic for rabbits (4) and vervet monkeys (5) when fed as part of a semipurified, cholesterol-free diet. However, when peanut oil is subjected to randomization (rearrangement), it becomes less atherogenic for cholesterol-fed rabbits (6) and vervet monkeys fed a semipurified, cholesterol-free diet (5). It was, therefore, suggested that the structure of the component triglycerides was as important to its atherogenicity as its fatty acid spectrum. Analysis of the triglyceride of the native and randomized peanut oils showed that the former had more triglycerides with linoleic acid in the 2-position and saturated fatty acids in the 1- and 3-positions (7). Randomization is a process in which the fatty acids of a fat are rearranged so as to provide a fat in which each component fatty acid is present in each triglyceride position to one-third of its total concentration.

There are many possible explanations for this interesting observation. One is that the absorption and transport of dietary cholesterol could be different in the presence of peanut oil (PNO) or randomized peanut oil (RPNO). The aim of this investigation was to study the absorption and transport of cholesterol into lymph in rats fed with cholesterol plus either peanut oil or randomized peanut oil.

*To whom correspondence should be addressed.

MATERIALS AND METHODS

Lymph Fistula Studies

Animals. Male Sprague-Dawley rats (250-275 g) were fasted overnight before the operation. With the animals under ether anesthesia, the intestinal (mesenteric) lymph duct was cannulated with a clear vinyl tubing (od, 0.8 mm), according to the method of Bollman et al. (8). Silicone tubing (od, 1.6 mm) tipped inside with a clear vinyl tubing (od, 1 mm) was introduced ca. 2 cm down the duodenum through the fundus of the stomach. The tubing was secured in the duodenum through a transmural suture, and the fundal incision was closed by a purse-string suture. During the whole recovery period, the animals were infused with 3 ml/hr of a saline solution (145 mM NaCl) containing KCl (4 mM) and glucose (0.28 M). The animals were allowed to recover for at least 36 hr in restraining cages kept in a warm chamber (~30 C) before the lipid infusion. Morgan (9) demonstrated that bile fistula rats absorbed lipid significantly better at 48 hr than at 24 hr after the operation.

Experimental plan. The control rats were infused with a lipid emulsion containing 30 μmol of peanut oil triglyceride, 3.9 μmol of cholesterol (containing [4-^{14}C]cholesterol) and 57 μmol sodium taurocholate in 3 ml of phosphate-buffered saline, pH 6.4. The experimental rats received the same lipid infusion except that the peanut oil was replaced by randomized peanut oil.

Preparation of infusate. On the day of the experiment, peanut oil was weighed in a beaker. Then an appropriate volume of a cholesterol stock solution containing [4-^{14}C]cholesterol in chloroform was added. The chloroform was evaporated under a stream of nitrogen. Sodium taurocholate (19 mM) dissolved in phosphate-buffered saline (6.75 mM Na_2HPO_4/16.5 mM NaH_2PO_4/115 mM NaCl/4 mM KCl) was then added to make the required lipid concentration, and the mixture was sonicated. Both the control and the experimental infusates formed a stable emulsion. The pH of the infusate was adjusted to 6.4 with 1 M NaOH. Samples of infusate were analyzed for glyceride ester and cholesterol radioactivity at the beginning and end of infusion (reproducibility ± 5%).

Experimental Procedure

Lymph was collected for 2 hr before lipid infusion and analyzed as the fasting lymph. Additional lymph samples were collected hourly during the 6-hr infusion period. At the end of infusion, animals were anesthetized and killed by exsanguination. The upper and the lower halves of the small intestine were tied off separately and the contents of each were eluted twice with 5-ml aliquots of 5 mM sodium taurocholate. Aliquots were taken from these washings for radioactivity determination by using a water-miscible scintillant, "Aquasolv" (Beckman, Fullerton, CA).

The stomach and the colon were excised separately with care, to prevent leakage of luminal contents, and put into stoppered Erlenmeyer flasks. All feces passed after the rats received the test meal were added to the colonic sample. Samples were saponified, acidified, and extracted with petroleum ether (10). A sample of the petroleum ether phase was taken for quantitation of the ^{14}C-lipid content by liquid scintillation spectrometry.

Mucosa from the upper and lower small intestinal wall was scraped with glass slides and lipids were extracted with toluene/ethanol (2:1, v/v) (11). Aliquots were taken for both radioactivity determination in the toluene-based scintillant and also for the determination of the composition of the fatty acids present.

Lymph lipid was extracted by the method of Folch et al. (12). Aliquots were taken for determination of esterified fatty acid (13), phospholipid (14) and cholesterol (15). Aliquots were also taken for measurement of radioactivity and the determination of the fatty acid by gas liquid chromatography (GLC).

Materials. [4-^{14}C]cholesterol was purchased from Amersham Corp., Arlington Heights, IL, and was purified by thin layer chromatography (TLC) before use. The native peanut oil of North American origin was a gift from Standard Brands, Wilton, CT, and had a fatty acid composition of 16:0-7.4, 18:0-1.2, 18:1-61.2, 18:2-24.8, 18:3-1.0, 20:0-1.1, 22:0-3.0 (4). Native peanut oil was subjected to a rearrangement of fatty acids to yield the randomized peanut oil with fatty acid and iodine value identical to that of the starting material (7). To check that randomization had occurred, both chromatographic and stereospecific analyses were performed and fatty acid was present in each position at 33% of its concentration in the total oil. Sodium taurocholate was supplied by Calbiochem-Behring, LaJolla, CA, and analyzed by TLC using a propionic acid/isoamyl acetate/water/*n*-propanol system (15:20:5:10, v/v). Cholesterol was supplied by Sigma Chemical, St. Louis, MO. All reagents used were of analytical grade.

Determination of the fatty acid composition of intestinal lymph lipid and mucosal lipid. The lipid from the intestinal lymph and the intestinal mucosa were converted into methyl esters using 1 N sodium methoxide in methanol/benzene (60:10, v/v) as described by Glass (16). Fatty acid analysis was carried out using a Hewlett-Packard Gas Chromatograph Model 5830A with a 6-ft column of 10% SP2300 on 80/100 Supelcoport (Supelco, Bellefonte, PA).

RESULTS

Lymph Flow

Four rats were studied in each group. The fasting lymph flow was similar in both groups, 2.20 ± 0.33 ml/hr (mean ± SE) for those fed peanut oil (PNO) and 2.15 ± 0.24 ml/hr for those fed randomized peanut oil (RPNO). During the period of lipid infusion, the lymph flow rate increased significantly in both groups of rats to reach a steady output of 3.2-3.7 ml/hr during the 5th and 6th hr. No significant difference in lymph flow rate was detected between the 2 groups.

Lymphatic Transport of Triglyceride

The fasting triglyceride output was 4.01 ±

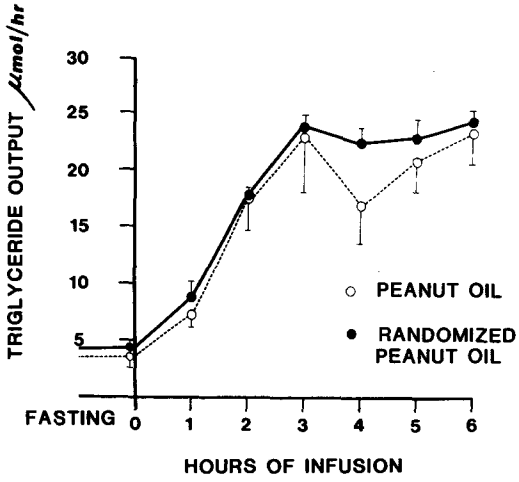

FIG. 1. Lymphatic triglyceride output. Output of triglyceride in lymph in μmol/hr was measured 2 hr before lipid infusion and also during the 6-hr lipid infusion. Results are expressed as mean ± standard error of the mean (4 PNO and 4 RPNO).

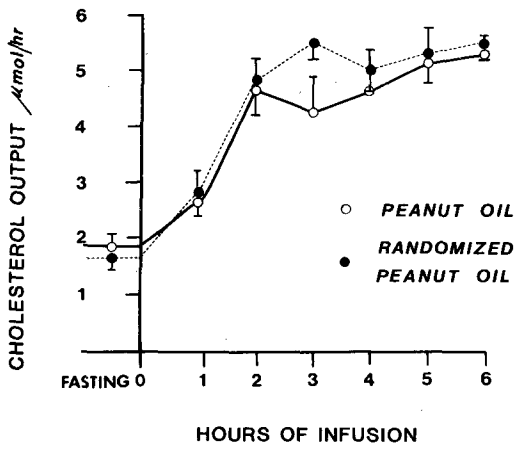

FIG. 2. Lymphatic cholesterol output. Cholesterol output in lymph in μmol/hr, as determined chemically, was measured 2 hr before lipid infusion and also during the 6-hr lipid infusion. Results are expressed as mean ± standard error of the mean (4 animals in each group). Although not shown in this figure, the radioactive cholesterol output data agreed well with the chemical output data.

0.58 μmol/hr (mean ± SE) for the PNO animals and 4.27 ± 0.14 μmol/hr for the RPNO animals. The triglyceride output increased in both groups after lipid infusion and reached a steady output of 23.36 ± 2.56 μmol/hr for the control and 24.24 ± 0.93 μmol/hr for the experimentals (Fig. 1). No significant difference in triglyceride output was detected between the 2 groups at any period during the 6 hr of lipid infusion.

Cholesterol

Cholesterol output into lymph was measured both radioactively and chemically. Because the chemical and the radioactivity data agreed well with each other, only the chemical data are presented in Figure 2. The fasting cholesterol output was 1.85 ± 0.24 μmol/hr (mean ± SE) for the PNO and 1.65 ± 0.19 μmol/hr for the RPNO rats. Cholesterol output increased significantly after lipid infusion in both groups and reached a steady output (5th and 6th hr) of 5.21 ± 0.17 μmol/hr in the PNO and 5.35 ± 0.20 μmol/hr for the RPNO rats. No significant difference was detected in cholesterol output between the 2 groups. Of the cholesterol transported in lymph, 70-80% was in the form of cholesteryl ester.

Phospholipid Output

During fasting, the phospholipid output was 1.34 ± 0.19 μmol/hr for the PNO and 1.00 ± 0.36 μmol/hr for the RPNO rats. The phospholipid output increased concomitantly with the increase in cholesterol and triglyceride output

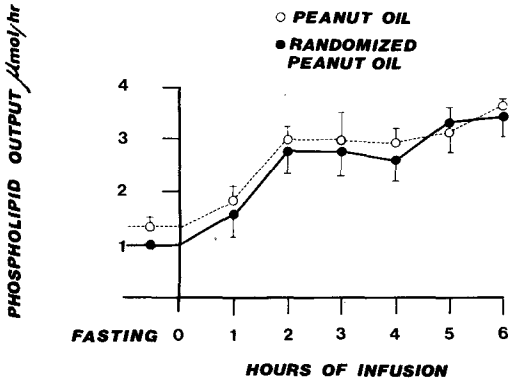

FIG. 3. Lymphatic phospholipid output. Output of phospholipid in lymph in μmol/hr was measured 2 hr before lipid infusion and also during the 6-hr lipid infusion. Results are mean ± standard error of mean for groups of 4 rats.

and appeared to be reaching a plateau during the 5th and 6th hr (Fig. 3). The phospholipid output during the 6th hr for the PNO was 3.63 ± 0.10 μmol/hr and 3.41 ± 0.37 μmol/hr for the RPNO rats.

Luminal and Mucosal Recovery of Labeled Cholesterol

Recovery of ^{14}C-cholesterol in the gastrointestinal luminal contents showed that uptake of radioactive cholesterol was similar in both the PNO and RPNO rats (Fig. 4). In both groups, ca. 8-9% of the infused radioactive

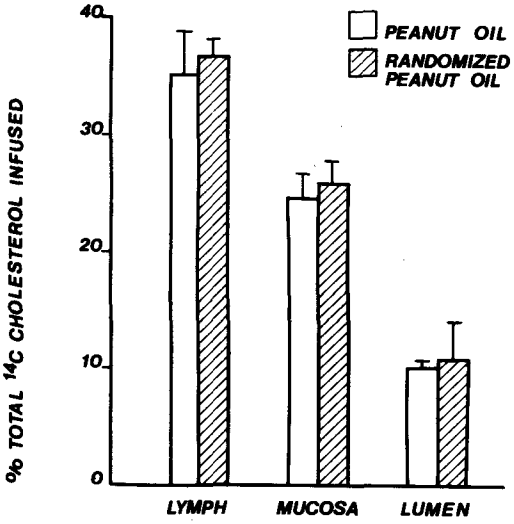

FIG. 4. Recovery of infused [^{14}C] cholesterol. At the end of 6 hr of [^{14}C] cholesterol infusion, amount of radioactive lipid (as a percentage of total infused dose) was measured in lumen of gastrointestinal tract and mucosa of small intestine. Also shown is recovery of [^{14}C] cholesterol in lymph collected in lymph over 6-hr infusion period. Values are mean ± standard error of the mean.

cholesterol remained unabsorbed in the gastrointestinal lumen. The distribution of the radioactive cholesterol between the upper and the lower intestinal lumen showed no difference in the small intestinal transit between the 2 groups of rats. Of the lipid absorbed, 24.4% of the total infused dose remained in the small intestinal mucosa in the PNO rats. In the RPNO rats, 25.7% of the total infused dose was recovered from the small intestinal mucosa. Of the recovered mucosal ^{14}C-cholesterol, 80% and 87% were found in the upper small intestinal mucosa of the PNO and the RPNO rats, respectively. This difference is not statistically significant.

As shown in Figure 4, the total ^{14}C-cholesterol output into lymph during the 6 hr when expressed as percent of total dose infused was similar in both groups of rats (35.4% in the PNO and 37.1% in the RPNO animals). Thus, the presence of either peanut oil or randomized peanut oil did not affect the transport of cholesterol from the enterocytes into lymph as chylomicrons.

The total amount of cholesterol recovered in lumen, lymph, and mucosa from both groups was similar (69.7% ± 2.4% for control and 73.4 ± 4.4% for experimental rats).

Fatty Acid Composition of Lymph and Mucosal Lipid

The fatty acid composition of lymph lipid is summarized in Table 1. The major fatty acids present in the 4th and 5th hr intestinal lymph of both PNO and RPNO rats were palmitate, oleate and linoleate. No significant difference was detected between the intestinal lymph fatty acid composition of the PNO and the RPNO rats at the 5th hr of lipid infusion. Both arachidic and behenic acids were present in intestinal lymph of both groups of rats. How-

TABLE 1

Fatty Acid Composition of the 5th hr Lymph Lipid (Mass Percent)[a] of Rat Fed Peanut Oil (PNO) or Randomized Peanut Oil (RPNO)

	PNO Group 5th hr	RPNO Group 5th hr
12:0	ND[b]	ND[b]
14:0	trace	trace
16:0	17.70 ± 1.12	18.30 ± 0.98
16:1	0.93 ± 0.12	0.30 ± 0.30
18:0	4.70 ± 0.23	4.40 ± 2.04
18:1	36.90 ± 0.92	36.10 ± 4.88
18:2	30.20 ± 0.77	29.10 ± 0.25
18:3	1.30 ± 0.31	1.50 ± 0.15
20:0	0.93 ± 0.23	0.27 ± 0.27
20:1	1.80 ± 0.41	1.10 ± 0.58
20:4	3.60 ± 0.41	4.00 ± 1.98
22:0	1.80 ± 0.35	1.40 ± 0.33
24:0	0.80 ± 0.40	trace

[a]Values are expressed as mean ± SE. Three animals' intestinal lymph was analyzed for each time point.

[b]ND = not detected.

TABLE 2

Fatty Acid Composition of the Intestinal Mucosal Lipid (Mass Percent)[a] of Rats Fed Peanut Oil (PNO) or Randomized Peanut Oil (RPNO)

	PNO Group	RPNO Group
12:0	0.06 ± 0.03	0.10 ± 0.06
14:0	0.80 ± 0.20	0.80 ± 0.20
16:0	16.60 ± 0.75	16.70 ± 0.70
16:1	1.50 ± 0.32	1.60 ± 0.39
18:0	7.60 ± 0.57	7.20 ± 0.70
18:1	29.10 ± 2.44	32.50 ± 0.09
18:2	33.90 ± 2.04	31.10 ± 1.10
18:3	0.60 ± 0.15	0.60 ± 0.12
20:0	0.70 ± 0.35	0.60 ± 0.12
20:1	1.10 ± 0.12	1.00 ± 0.07
20:2	0.17 ± 0.03	0.17 ± 0.03
20:3	0.20 ± 0.15	0.40 ± 0.31
20:4	4.70 ± 0.48	4.60 ± 1.53
22:0	0.90 ± 0.33	1.10 ± 0.49
22:3	ND[b]	ND[b]
22:6	0.20 ± 0.03	0.20 ± 0.03
24:0	0.30 ± 0.15	0.30 ± 0.09

[a]Values are expressed as mean ± SE. Three animals' mucosal lipid was analyzed.

[b]ND = not detected.

ever, they were present in smaller concentrations than in the lipid infused. Also supporting these data was the fatty acid composition of intestinal mucosal lipid from the control and the experimental rats (Table 2). Similar to the lymph lipid fatty acids, palmitate, oleate and linoleate were the major fatty acids present in the intestinal mucosa. No real difference could be detected between the mucosal lipid fatty acids between the 2 groups.

DISCUSSION

The atherogenicity of peanut oil is reduced when the structure of the oil is altered by randomization. One of the possible explanations for this marked difference between the atherogenicity of native peanut oil and the randomized peanut oil could be the difference in the amount of cholesterol transported into lymph by the intestinal epithelial cells (enterocytes) when either of the oils is present. This study was conducted to address this question.

When cholesterol (3.9 μmol/hr) was infused intraduodenally together with either the peanut oil or the randomized peanut oil, the lymph flow rate increased in both groups of rats. This observation of increased lymph flow rate after lipid infusion has been well documented by various investigators (17-19). No significant difference was observed between the 2 groups of rats in lymph flow during the entire lipid infusion period.

The fasting triglyceride output was 4.01 μmol/hr for the PNO and 4.27 μmol/hr for the RPNO rats. The fasting triglyceride output observed in this study is comparable to those observed in previous studies (20). Both the fasting cholesterol output (1.85 μmol/hr for the PNO and 1.65 for the RPNO rats) and phospholipid output (1.34 μmol/hr for PNO and 1.00 μmol/hr for RPNO rats) are comparable to those observed in other studies (21,22). The output of triglyceride, phospholipid and cholesterol increased after lipid infusion and no significant difference in their outputs was observed during the entire lipid infusion period between the 2 groups of rats. Therefore, the difference in the atherogenicity of peanut oil and the randomized peanut oil is not due to a difference in the amount of cholesterol transported into lymph. Rather, this difference is caused by events subsequent to the transport of cholesterol into lymph by the small intestine, e.g., lipoprotein structure and its metabolism. However, it is important to note that the rat was not the original animal model in which the difference in atherogenicity between peanut oil and randomized peanut oil was demonstrated. Although it is more difficult to carry out the reported studies in rabbits and monkeys, it is important to confirm the present observation in these animals.

The lymphatic lipid output data are further supported by the observation of the recovery of infused ^{14}C-cholesterol from the different gastrointestinal segments. In both groups of rats, ca. 10% of the total infused ^{14}C-cholesterol was recovered in the gastrointestinal lumen. Of the 90% of ^{14}C-cholesterol absorbed by the small intestine of both groups of rats, ca. 25% of the ^{14}C-cholesterol still remained in the small intestinal mucosa. This observation again confirmed that there is no difference in the absorption and transport of cholesterol in the presence of either peanut oil or randomized peanut oil.

The fatty acid composition of intestinal lymph lipid is similar in both groups of rats. This is also true for mucosal lipids. Therefore, it would seem that the metabolism of the peanut oil fatty acid by the enterocytes is similar to those derived from randomized peanut oil. However, our data do not rule out the possibility that there is a difference in the arrangement of the fatty acid in the various lipid fractions of intestinal lymph lipoproteins of the PNO and RPNO rats.

In conclusion, we have detected no difference in the effect of the native peanut oil and the randomized peanut oil on the absorption and transport of cholesterol by the rat small intestine. The difference in the atherogenicity of these 2 diets is probably caused by events subsequent to the entry of the cholesterol containing chylomicrons and very low density lipoproteins into plasma.

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REFERENCES

1. Gresham, G.A., and Howard, A.N. (1960) Br. J. Exp. Path. 41, 395-402.
2. Kritchevsky, D., Tepper, S.A., Vesselinovitch, D., and Wissler, R.W. (1971) Atherosclerosis 14, 53-64.
3. Vesselinovitch, D., Getz, G.S., Hughes, R.H., and Wissler, R.W. (1974) Atherosclerosis 20, 303-321.

4. Kritchevsky, D., Tepper, S.A., Kim, H.K., Story, J.A., Vesselinovitch, D., and Wissler, R.W. (1976) Exp. Molec. Pathol. 24, 375-391.
5. Kritchevsky, D., Davidson, L.M., Weight, M., Kriek, N.P.J., and duPlessis, J.P. (1982) Atherosclerosis 42, 53-58.
6. Kritchevsky, D., Tepper, S.A., Vesselinovitch, D., and Wissler, R.W. (1973) Atherosclerosis 17, 225-243.
7. Myher, J.J., Marai, L., Kuksis, A., and Kritchevsky, D. (1977) Lipids 12, 775-785.
8. Bollman, J.L., Cain, J.C., and Grindlay, J.H. (1949) J. Lab. Clin. Med. 33, 1349-1352.
9. Morgan, R.G.H. (1966) Q. J. Exp. Physiol. 51, 33-41.
10. Rodgers, J.B., Fondacaro, J.D., and Kot, J. (1977) J. Lab. Clin. Med. 89, 147-152.
11. Bennett-Clark, S. (1971) J. Lipid Res. 12, 43-55.
12. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 266, 497-509.
13. Stern, I., and Shapiro, B. (1953) J. Clin. Pathol. 6, 158-160.
14. Parker, F., and Peterson, N.F. (1965) J. Lipid Res. 6, 455-460.
15. Zak, B. (1957) Am. J. Clin. Pathol. 27, 583-588.
16. Glass, R.L. (1971) Lipids 6, 919-925.
17. Borgström, B., and Laurell, C.B. (1953) Acta Physiol. Scand. 29, 264-280.
18. Simmonds, W.J. (1955) Aust. J. Exp. Biol. Med. Sci. 33, 305-314.
19. Turner, S.G., and Barrowman, J.A. (1977) Q. J. Exp. Physiol. 62, 175-180.
20. Tso, P., Balint, J.A., and Simmonds, W.J. (1977) Gastroenterology 73, 1362-1367.
21. Tso, P., Balint, J.A., and Rodgers, J.B. (1980) Am. J. Physiol. 239, G348-G353.
22. Tso, P., Balint, J.A., Bishop, M.B., and Rodgers, J.B. (1981) Am. J. Physiol. 241, G487-G497.

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The Role of Calcium in the Regulation of Prostacyclin Synthesis by Porcine Aortic Endothelial Cells

A.R. WHORTON[a]*, C.E. WILLIS[a], R.S. KENT[a] and S.L. YOUNG[b], [a]*Divisions of Clinical Pharmacology, Cardiology and Pulmonary Medicine, Duke University Medical Center, Durham, NC 27710, and* [b]*Department of Medicine, Durham Veterans Administration Medical Center, Durham, NC 27705*

ABSTRACT

Both bradykinin (EC_{50} = 8 ng/ml) and the ionophore A23187 (EC_{50} = 3 × 10^{-7} M) potently stimulated arachidonate release and prostaglandin synthesis in porcine aortic endothelial cells. The response to each was completely dependent on extracellular Ca^{2+} (EC_{50} = 3 × 10^{-7} M); no role for intracellular Ca^{2+} was noted. The rapid Ca^{2+} influx prompted by either activator was consistent with the time course for arachidonate release. Whereas the arachidonate released in response to bradykinin was transient, that released in response to A23187 was more prolonged, and paralleled a continued influx of Ca^{2+}. Ca^{2+} entry elicited by bradykinin was mediated by channels which could not be blocked by verapamil. When Mn^{2+} was substituted for Ca^{2+}, no stimulation of prostacyclin synthesis was seen in response to A23187; however, the bradykinin response was unaffected. The mechanism of these effects was studied using doses of bradykinin or A23187 which resulted in increases in Ca^{2+} influx and prostacyclin synthesis of similar magnitude for each agonist. Under these conditions, trifluoperazine blocked elevated prostacyclin synthesis (ID_{50} = 5-6 × 10^{-6} M for each agonist). Trifluoperazine sulfoxide, however, was much less active. Pimozide inhibited bradykinin-stimulated prostacyclin synthesis at low doses (ID_{50} = 3 × 10^{-6} M). Trifluoperazine was much less effective against high doses of A23187 (4 × 10^{-6} M). These data suggest that arachidonate release and prostacyclin synthesis are dependent on influx of extracellular calcium and subsequent activation of a Ca^{2+}-dependent phospholipase by a calmodulin-mediated mechanism.
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Although it is generally accepted that calcium is necessary for prostaglandin synthesis, its kinetics and mechanisms of action are largely uninvestigated (1,2). The major role proposed for calcium is activation of phospholipase A_2 and specific release of arachidonic acid (3). Evidence for control of this enzyme by calcium comes primarily from experiments using an ionophore, A23187 (3,4), although calcium-dependent phospholipase activation has also been proposed for hormones such as thrombin (4,5) and bradykinin (6,7). The similarity between A23187 and hormone-stimulated arachidonic acid release is questionable in view of experiments in platelets which demonstrate differential activation of distinct phospholipases (8). In other studies on platelets, a calcium-dependent, phosphoinositide-specific phospholipase C has been shown to be important in the initial step of arachidonate release (9) and, although still controversial, the source of the Ca^{2+} appears to be intracellular stores (10). The phospholipase activated in other cells by calcium appears to be phospholipase A_2 (11). The source of calcium may be intracellular as in endothelial cells (5) or extracellular as in cells derived from renal tissue where calcium enters through voltage-dependent channels (12).

We and others have suggested that the intracellular effects of calcium are, in fact, mediated by a calcium-calmodulin complex (7,13). In an earlier study, we showed that a phospholipase in cultured endothelial cells is activated by a calcium-calmodulin-dependent pathway after stimulation of cells with bradykinin (7). In the present study, we report further data to support the role of calmodulin in phospholipase activation, and explore the generality of this mechanism using other agonists such as A23187. To describe more completely the role of calcium, we have also investigated both the dose-dependency and kinetics of calcium in hormone and ionophore-stimulated prostacyclin synthesis, as well as the source of calcium for these effects.

MATERIALS AND METHODS

Bradykinin triacetate was purchased from Sigma Chemical Co. (St. Louis, MO), ionophore A23187 (free acid) from Calbiochem (La Jolla, CA), 5,6,8,9,11,12,14,15-[^{3}H]arachidonic acid and $^{45}Ca^{2+}$ from New England Nuclear (Boston, MA), trifluoperazine and trifluoperazine sulfoxide from Smith, Kline, and French (Philadel-

*To whom correspondence should be addressed.

phia, PA), pimozide from McNeil Pharmaceuticals (Spring House, PA), verapamil from Knoll Pharmaceuticals (Whippany, NJ), and culture media and buffers from Grand Island Biological Co. (Grand Island, NY). All compounds were dissolved in incubation buffer immediately prior to an experiment. A23187 was dissolved in ethanol (2 mg/ml) and diluted with incubation buffer before use. The final ethanol concentration was less than 30 ppm for 10^{-7} M A23187 and had no demonstrable effect on the cells.

Endothelial cells were collected from porcine aortae and cultured as described by Jaffe et al. (14). Briefly, freshly collected aortae were cleaned, filled with 0.1-0.2% collagenase (clostridium perfringens, Sigma) in medium 199, sealed with clamps and incubated at 37 C for 15-20 min. Freed cells were collected, washed by centrifugation at 4 C and resuspended in medium 199 containing Hank's salts, 20% fetal calf serum, and antibiotic/antimycotic mixture. Cells were then plated (400,000/flask) in 25-cm^2 polystyrene flasks (Costar, Cambridge, MA) and monitored until ca. 50% of the cell clumps adhered. Nonadhering cells were poured off and the primary isolates incubated in medium 199 plus 20% fetal calf serum at 37 C. The medium was changed every 3 days and the fetal calf serum was progressively reduced to 1% as the cells grew to confluence. Porcine endothelial cells in culture were polygonal in shape and demonstrated contact inhibition. Further identification was as previously published (7). Primary and subsequent cultures were treated with 0.1% EDTA at 37 C and split 1:4 for subculture. In general, confluent cultures in the first or second passage were used, although cells have been studied after the fifth passage with no apparent difference in response. Except where noted, all incubations were carried out in air at 37 C in Hank's balanced salt solution at pH 7.4. In experiments where inhibitors were to be tested and ion concentrations varied, cells were preincubated 5 min prior to addition of agonists.

To study the calcium requirement for prostaglandin synthesis, endothelial cells at confluence were washed and preincubated in Hank's balanced salt solution containing varying Ca^{2+} concentrations. After 10 min, the preincubation buffer was collected and analyzed for PGI_2 to establish baseline synthesis under these conditions. Fresh buffer with the appropriate external Ca^{2+} concentration and with agonist (BK or A23187) was then added and the incubation continued for 10 min. Again, the buffer was collected and analyzed for PGI_2 production.

To study release of labeled compounds stimulated by agonists, endothelial cells at confluence were labeled by incubation with 2 μCi of [^{3}H]arachidonic acid (sodium salt; 60-100 Ci/mmol) for 3-4 hr. After the labeling period, cells were washed by replacing the medium with fresh medium 199. The cells were then incubated for an additional 30 min, after which the medium was replaced with medium containing 0.5% bovine serum albumin (fatty acid free, Sigma). Aliquots were taken at times indicated to establish baseline values, and again after bradykinin or ionophore A23187.

To study calcium influx, cells were incubated in Hank's balanced salt solution containing $^{45}Ca^{2+}$, either in the absence (control) or presence of bradykinin or A23187 to stimulate influx. At the times indicated, the media was quickly poured off and the cells washed 3 times with ice-cold buffer. Cells were then denatured and intracellular Ca^{2+} solubilized by addition of 1 ml of 6% trichloroacetic acid. Aliquots were then used for determining $^{45}Ca^{2+}$ using a liquid scintillation counter.

Prostacyclin, PGI_2, released by cells after incubation was measured as its stable hydrolysis end-product, 6-keto-$PGF_{1\alpha}$, and quantitated by gas chromatography-mass spectrometry (GC-MS) employing selected ion monitoring (15, 16). After addition of a deuterated prostaglandin analogue internal standard (3,3,4,4-[^{2}H]-6-ketoprostaglandin $F_{1\alpha}$, Upjohn), the samples were acidified to pH 3 with formic acid and extracted twice with 2 ml ethyl acetate. Before analysis by mass spectrometry, prostaglandins were methylated (diazomethane), silylated (N,O-*bis* (trimethylsilyl) trifluoroacetamide) and methoxylated (methoxamine-HCL) as previously described (16). Selected ion monitoring was done using a quadrapole gas chromatograph-mass spectrometer (Hewlett Packard 5992-B) equipped with a 3-ft glass column packed with 3% OV-1 on 80/100 mesh Chromosorb WHP and operated isothermally at 230 C with helium as the carrier gas (22 cc/min). Ion pairs monitored were (m/z) 598 vs 602 for 6-keto-$PGF_{1\alpha}$.

RESULTS

We have previously reported that both bradykinin and the ionophore A23187 potently stimulate PGI_2 synthesis in cultured porcine aortic endothelial cells (7). The response to bradykinin was found to be dose-dependent (ED_{50} = 8 ng/ml) with the maximum stimulation of PGI_2 synthesis being 3-fold at 100 ng/ml (7). As shown in Figure 1, the log dose-response relationship for A23187 was relatively

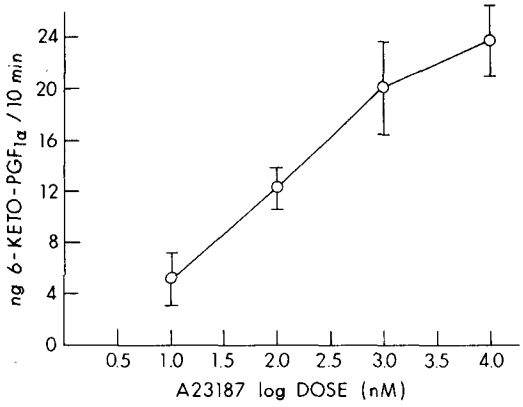

FIG. 1. Log dose relationship between A23187 and prostacyclin production by aortic endothelial cells. Cells (1-1.5 X 10^6 cells) at confluence (1st to 4th passage) were incubated in Hank's balanced salts (no albumin), pH 7.4 at 37 C, under an air atmosphere with varying concentrations of A23187. The medium was removed after 10 min and analyzed for PGI_2 by GC-MS. Data given as mean ± SD, n = 9.

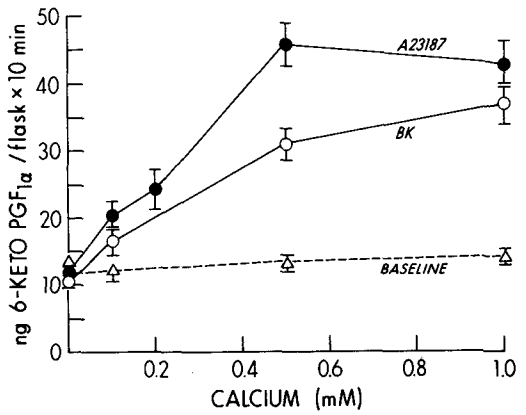

FIG. 2. Effect of extracellular Ca^{2+} on both bradykinin (100 ng/ml) and A23187 (10^{-7} M)-stimulated PGI_2 release. Cells were incubated as in Figure 1 in buffers containing varying Ca^{2+} concentrations. The medium was removed after 10 min and analyzed for baseline PGI_2 production by GC-MS. Fresh buffer containing the appropriate Ca^{2+} concentration and agonist was then added. After 10 min, the medium was removed and analyzed for PGI_2 production. Data given as mean ± SEM, n = 4.

linear from 10^{-8} to 10^{-5} M with an ED_{50} of ca. 3 x 10^{-7} M. At 10^{-5} M, the stimulation of PGI_2 formation was 7-fold above control (3.5 ± 0.2 ng/10 min), whereas at 10^{-7} M A23187 the response was closer in magnitude to the maximum bradykinin-elicited response (3-fold). The responses to both A23187 (10^{-7} M) and bradykinin (100 ng/ml) were dependent on extracellular Ca^{2+}, and as shown in Figure 2, both dose-response curves demonstrated an EC_{50} of ca. 0.3 mM. At zero external calcium concentration, neither bradykinin nor A23187 were able to stimulate PGI_2 synthesis above baseline values.

We further examined the mechanism of action of bradykinin and A23187 by studying the kinetics of arachidonic acid release from cells in culture. Endothelial cells were incubated for 3 hr with [^{3}H]arachidonic acid, washed and used to investigate arachidonate release. In these experiments, albumin was added to the buffer to trap the released fatty acids. Under these conditions, essentially all of the label released was ^{3}H-arachidonate (82%) (7). When albumin was omitted from the buffer (as it was for all other experiments), ^{3}H-arachidonate accounted for 43% of label released; the rest was metabolized to prostaglandins as previously reported (7). The data in Figure 3 represent the rate of release of radioactivity (primarily arachidonate) following stimulation of labeled cells with either bradykinin or A23187 (added at t = 30 min). Both agonists elicited a peak response by 5 min. However, whereas the response to bradykinin had essentially returned to the baseline by 10 min, the response to A23187 had decreased by only 50% of maximum at 10 min and was

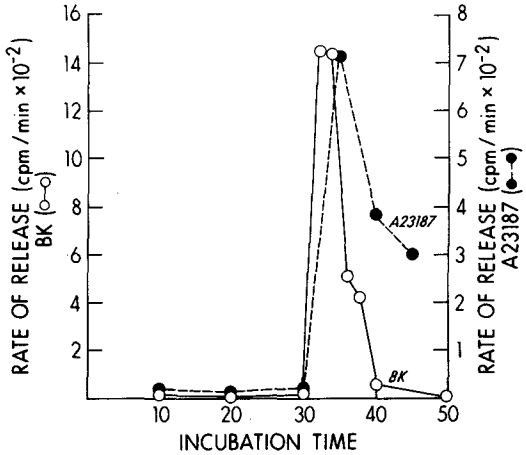

FIG. 3. Rate of release of labeled compounds from endothelial cells prelabeled with [^{3}H]arachidonic acid. Cells were grown to confluence and incubated with [^{3}H]arachidonic acid (2 μCi–ca. 8 ng) for 3-4 hr. The labeled cells were then washed as described in the text and the control period started. Samples were taken at 0, 10, 20 and 30 min and assayed for label released. Either bradykinin (500 ng/ml) or A23187 (10^{-7} M) was added at t = 30 min and additional samples taken. Triplicate data calculated as change in radioactivity in the medium per each time period (cpm/min).

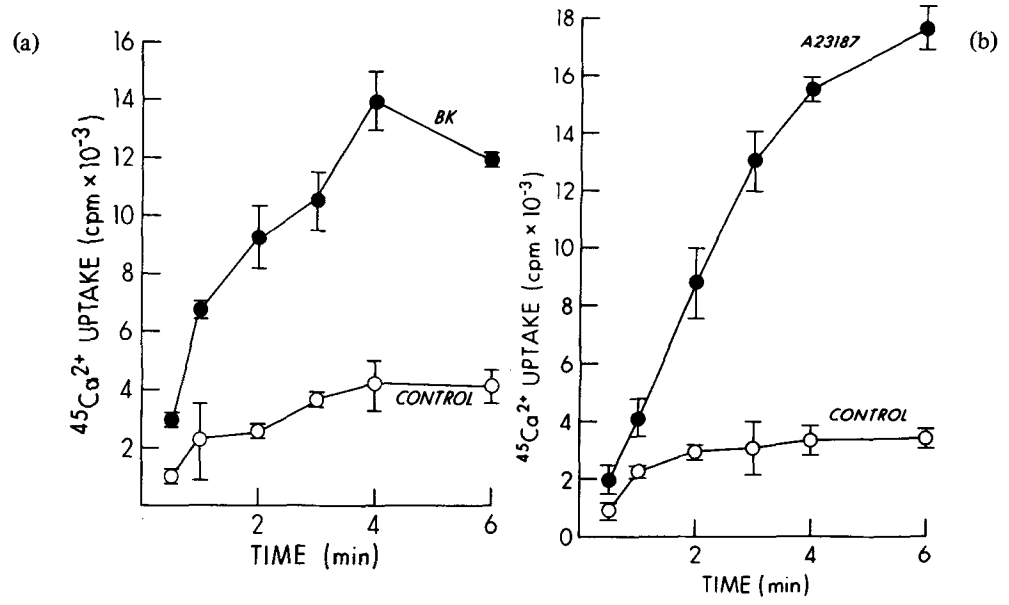

FIG. 4. (A) Effect of bradykinin (100 ng/ml) on $^{45}Ca^{2+}$ uptake into endothelial cells grown in culture. Cells at confluence were incubated for the times indicated with 2 ml of buffer containing $^{45}Ca^{2+}$ (10 μCi, 1.26 mM) with or without bradykinin. The incubation was terminated by rapidly removing the radioactive medium and washing with ice-cold nonradioactive medium. Cells were then lysed and the intracellular Ca^{2+} solubilized by addition of 1 ml of 6% trichloroacetic acid. $^{45}Ca^{2+}$ was determined using a liquid scintillation counter. Each point represents mean ± SEM, n = 4. (B) Effect of A23187 (10^{-7} M) on $^{45}Ca^{2+}$ uptake. Experiment done as in Fig. 4A.

still 42% of maximum after 15 min. This prolonged action of A23187 is consistent with its ionophoretic properties. With time, the ionophore continues to deliver Ca^{2+} intracellularly. Thus, the extent of arachidonic acid release is most likely limited by substrate availability (pool size). The effects of bradykinin, however, are more transient and subject to mechanisms which regulate intracellular Ca^{2+} concentrations. The data in Figures 4A and 4B confirm this. For these experiments, cells were incubated in media containing $^{45}Ca^{2+}$. The baseline influx of this ion was determined and compared to that found after addition of either bradykinin or A23187. Both compounds increased Ca^{2+} influx with a time course consistent with the stimulation of arachidonate release (Fig. 3). As expected, the $^{45}Ca^{2+}$ influx induced by A23187 continued for at least the first 6 min. These data, as well as those in Figures 2 and 6 (see below), strongly suggest complete dependence on external Ca^{2+} supplies. However, when cells were pretreated with verapamil (5×10^{-6} M), no effect was seen on bradykinin-stimulated PGI_2 synthesis (Fig. 5). In fact, as can be seen in Table 1, verapamil was not effective in blocking BK-induced $^{45}Ca^{2+}$ influx and, as one might expect since A23187 creates its own "channels," verapamil was not effective in blocking A23187-induced $^{45}Ca^{2+}$ influx. Thus, channels other than those blocked by verapamil must be utilized in this response.

In view of these data, we felt it was important in subsequent experiments to study the mechanism by which these compounds elevate PGI_2 synthesis using doses of each which resulted in comparable net changes in intracellular Ca^{2+} levels, i.e., 3-fold stimulation of PGI_2 synthesis (100 ng/ml bradykinin and 10^{-7} M A23187).

The absolute dependence of arachidonate release on calcium was further studied. A23187, although it binds Ca^{2+} effectively, binds Mn^{2+} ca. 100 times more tightly. Thus, Mn^{2+} should block the actions of A23187 even in the presence of calcium. The data in Figure 6 show that Mn^{2+} effectively competes for and prevents the actions of A23187 while having no effect on either control or bradykinin effects. Further, from the data in Table 1, it can be seen that Mn^{2+} prohibits A23187-induced $^{45}Ca^{2+}$ influx. In addition, the data in Figure 6 (as those in Fig. 2) demonstrate the requirement for external Ca^{2+} for agonist-induced activity. Thus,

TABLE 1

The Effects of Verapamil and Mn^{2+} on $^{45}Ca^{2+}$ Influx into Endothelial Cells

Condition	$^{45}Ca^{2+}$ Influx (cpm)[a]
Control	1335 ± 102
A23187 (10^{-7} M)	5366 ± 170[b]
A23187 + verapamil (5×10^{-6} M)	5645 ± 299[c]
A23187 + Mn^{2+} (0.126 mM)	2010 ± 74[d]
BK (100 ng/ml)	4873 ± 178[b]
BK + verapamil (5×10^{-6} M)	4591 ± 206[c]

[a] $^{45}Ca^{2+}$ uptake was determined after incubating cells for 5 min with 5 μCi of $^{45}Ca^{2+}$ plus additions as listed. Cells were treated as in Figure 4. Data given as mean ± SEM, n = 4. Data analyzed by analysis of variance. Multiple comparisons by Student-Newman-Keuls test (19).

[b] $p < 0.01$ as compared to control.

[c] Not different from agonist alone.

[d] $p < 0.01$ as compared to A23187 alone. Not different from control.

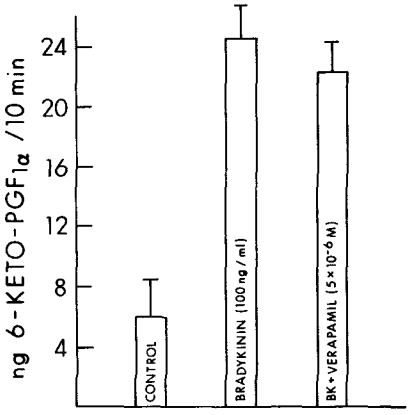

FIG. 5. Effect of verapamil (5×10^{-6} M) on bradykinin (100 ng/ml)-stimulated PGI_2 synthesis in endothelial cells. Experiment done as in Fig. 1. Verapamil was preincubated with cells for 5 min before the addition of bradykinin. Data given as mean ± SEM, n = 6. Although verapamil did not significantly block the bradykinin effect, both bradykinin and verapamil + bradykinin-stimulated PGI_2 synthesis were statistically greater than control ($p < 0.01$, one-way analysis of variance and Student-Newman-Keuls test [19]).

the ionophore A23187 acts very specifically and selectively on these cells causing a response due only to calcium influx. These data and those in Figure 2 suggest the critical importance of extracellular calcium in phospholipase activation and provide no evidence to implicate intracellular Ca^{2+} in this process.

Since both bradykinin and A23187 act through Ca^{2+}-dependent mechanisms, and as we had previously suggested a role for calmodulin in the bradykinin response (7), we sought to determine if both bradykinin and A23187 act through a common calmodulin-mediated pathway. Using trifluoperazine, a compound which binds to and blocks the actions of calmodulin (17), we found a dose-dependent inhibition of both bradykinin (100 ng/ml) and A23187 (10^{-7} M)-stimulated PGI_2 synthesis. The ID_{50} of trifluoperazine against both agonist was ca. $5\text{-}6 \times 10^{-6}$ M, suggesting that bradykinin and A23187 both utilize the same calmodulin-dependent pathway (Fig. 7). Trifluoperazine sulfoxide, which binds much less efficiently to calmodulin (17), inhibited A23187 stimulation by a maximum of 35% at 8.0×10^{-5} M and inhibited bradykinin by 20% at the same dose (Fig. 8). Pimozide, a compound which binds to and inhibits calmodulin more effectively than trifluoperazine and is structurally unrelated to the phenothiazines (17), inhibited bradykinin-stimulated PGI_2 synthesis with an ID_{50} of 3.2×10^{-6} M (Fig. 9): a finding consistent with its 2-fold tighter binding to calmodulin than trifluoperazine ($ID_{50} = 6 \times 10^{-6}$ M). Interestingly, trifluoperazine was much less effective against high doses of A23187 (4×10^{-6} M) (Fig. 10), presumably due to the overwhelming influx of Ca^{2+} elicited by this compound.

DISCUSSION

In these experiments, we have studied the role of Ca^{2+} in prostacyclin synthesis in porcine aortic endothelial cells. Although a Ca^{2+} requirement has been proposed for prostaglandin synthesis in several systems, the exact nature of this requirement in terms of its kinetics and mechanism has not been studied. In addition, the source of Ca^{2+}, whether intracellular or extracellular, has remained controversial. In our studies on cultured endothelial cells, we have investigated these problems using 2 dissimilar means for activation of the pathway. The agonists used, bradykinin and A23187, stimu-

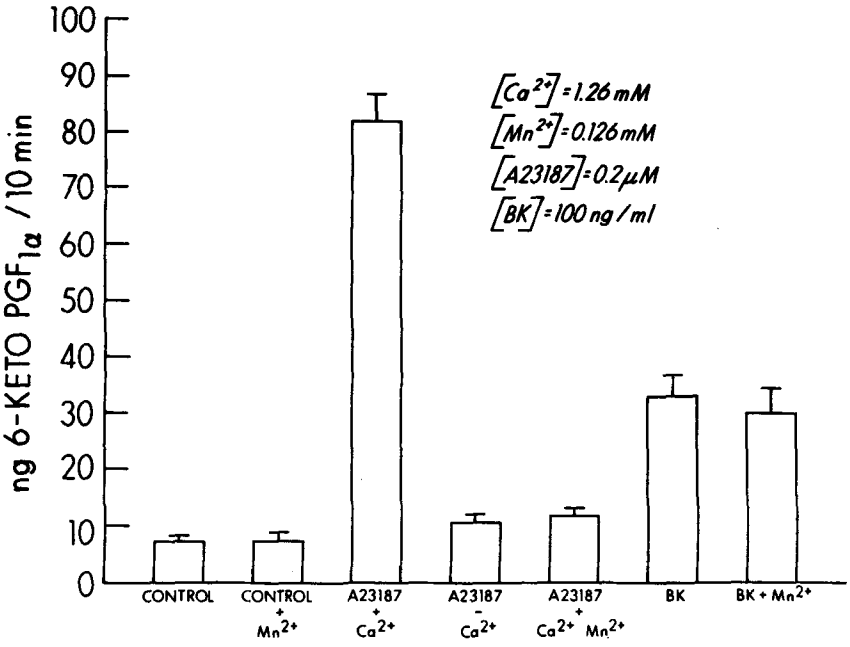

FIG. 6. Effect of Mn^{2+} and Ca^{2+} on A23187 (2×10^{-7} M) and bradykinin (100 ng/ml)-stimulated PGI_2 synthesis in endothelial cells. Cells at confluence were incubated in buffers with varying concentrations of Ca^{2+} and Mn^{2+}. After addition of either A23187 or bradykinin, cells were incubated for 10 min and the synthesis of PGI_2 determined by GC-MS. Data given as mean ± SEM, n = 5. A23187 + Ca^{2+}, BK, and BK + Mn^{2+} stimulations were significantly greater than control, $p < 0.01$; no other significant differences were noted (analysis of variance followed by Student-Newman-Keuls test [19]).

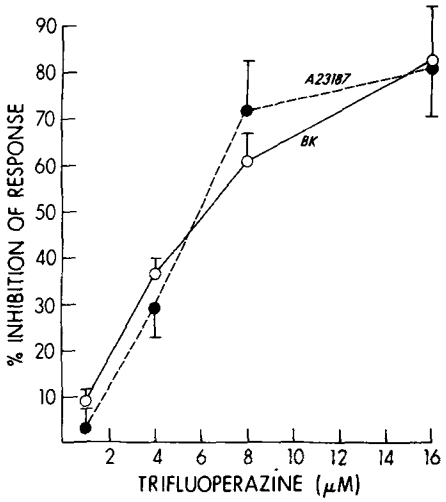

FIG. 7. Effect of trifluoperazine on A23187 (10^{-7} M) or bradykinin (100 ng/ml)-stimulated PGI_2 synthesis. Cells were incubated as in Fig. 1 for 5 min with or without trifluoperazine (13×10^{-6} M). The medium was then removed and replaced with buffer alone or buffer containing trifluoperazine. A23187 or bradykinin was added and the incubation continued for 10 min. Data are expressed as percentage inhibition of the maximum response to either agonist (mean ± SEM, n = 9 for bradykinin and n = 6 for A23187).

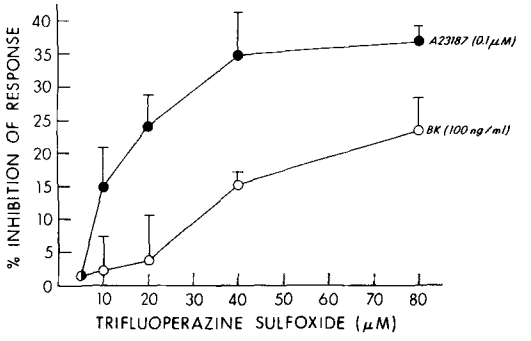

FIG. 8. Effect of trifluoperazine sulfoxide on A23187 (10^{-7} M) or bradykinin (100 ng/ml)-stimulated PGI_2 synthesis. Experiment done as in Fig. 7. Data given as percentage inhibition of the maximum response to either agonist (mean ± SEM, n = 4).

lated arachidonic acid release and PGI_2 production in a dose-dependent manner. These effects were absolutely dependent on the entry of extracellular calcium into the cells through verapamil-insensitive channels. These data are in contrast to those previously reported by others (5). The reason for this discrepancy is not clear. The time course of $^{45}Ca^{2+}$ influx into cells stimulated by either agonist was consistent

with the time course of arachidonic acid release. In contrast to the transient nature of bradykinin-stimulated arachidonic acid release and Ca^{2+} influx, the response to A23187 was sustained by continued Ca^{2+} influx. In fact, after stimulation with A23187, prostaglandin synthesis in these cells is most likely limited only by the size of the phospholipid pool serving as substrate for phospholipase A_2. Hormone-stimulated release is under more control. For example, in the case of bradykinin, the duration of the response is influenced by degradation of bradykinin (kinase II activity) and by mechanisms which control intracellular Ca^{2+} levels. Our data suggest that the increase in intracellular Ca^{2+} in response to bradykinin is transient and that the rate of Ca^{2+} influx begins to return to control by 6 min after stimulation. This phenomenon, in part, may reflect activation of the Ca^{2+}-ATPase ("calcium pump") by Ca^{2+}-calmodulin (18), with a resultant return of intracellular calcium to basal levels.

In a previous report (7), we suggested that the bradykinin response is mediated by Ca^{2+}-calmodulin. In the present study, we have continued to explore this hypothesis by using doses of A23187 and bradykinin which resulted in comparable net changes in arachidonic acid release and Ca^{2+} influx. Under these conditions, trifluoperazine, a drug which binds efficiently to calmodulin and prevents its actions ($K_i = 1 \times 10^{-6}$ M) (17), inhibited the activation of phospholipase in response to both bradykinin and A23187 ($ID_{50} = 5\text{-}6 \times 10^{-6}$ M). This inhibition occurred at doses of trifluoperazine far below those associated with nonspecific effects (10^{-4} M). The specificity assigned to trifluoperazine inhibition is supported by the lack of effect of trifluoperazine sulfoxide, a structurally similar compound which inactivates calmodulin at much higher doses ($K_i = 30 \times 10^{-6}$ M) (17). Since bradykinin and A23187 elicit calcium influx through different mechanisms, the similar efficacy of trifluoperazine in blocking the effects of both compounds also strengthens the hypothesis that a common Ca^{2+}-calmodulin pathway is involved. A role for calmodulin in arachidonic acid release is further supported by the observation that pimozide, a compound structurally unrelated to trifluoperazine, and one which binds tightly to calmodulin ($K_I = 0.8 \times 10^{-7}$ M), effectively inhibited bradykinin-induced release.

The above findings are in contrast to those from other studies which suggest different mechanisms for A23187 and thrombin-stimulated arachidonic acid release in platelets (8). However, we have shown that high doses of A23187 (4×10^{-6} M) are unaffected by doses of trifluoperazine (13×10^{-6} M) which completely block the bradykinin (or A23187 at 10^{-7} M) response. Presumably, the large influx of Ca^{2+} caused by 4×10^{-6} M A23187 is able to overcome the inhibitory effects of trifluoperazine, either by directly activating phospholipase A_2 or by some other unknown mechanisms. It is interesting to note that most of the studies in the literature have used doses of A23187 in excess of 10^{-6} M.

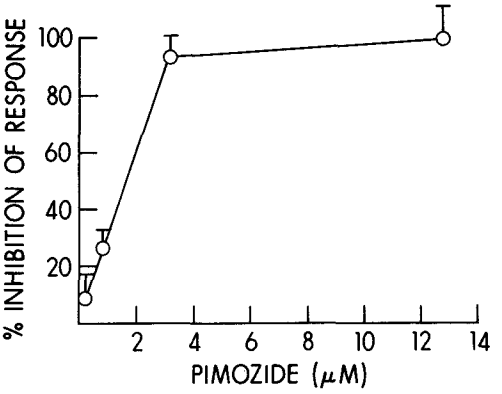

FIG. 9. Effect of pimozide on bradykinin-stimulated PGI_2 synthesis in endothelial cells. Experiment done as in Figure 7. Data given as percentage inhibition of the maximum response to bradykinin (mean ± SEM, n = 4).

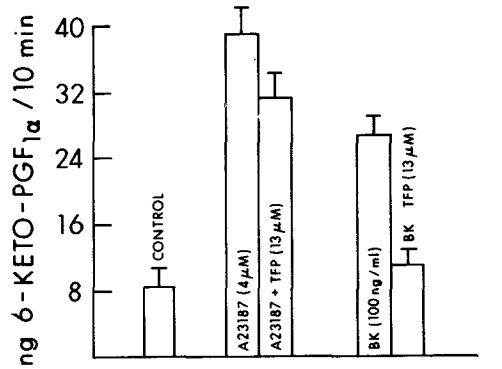

FIG. 10. Effect of trifluoperazine on high dose A23187 (4×10^{-6} M). Cells were incubated as in Fig. 7 with 13×10^{-6} M trifluoperazine. A23187 or bradykinin was used as agonist. Data given as total PGI_2 produced during 10 min (mean ± SEM, n = 5). The responses to A23187, A23187 + TFP, and BK were significantly greater than control ($p < 0.01$) and the response to BK was significantly greater than to BK + TFP ($p < 0.02$). No other significant differences were noted (analysis of variance followed by Student-Newman-Keuls test [19]).

Thus, it appears that prostaglandin synthesis in endothelial cells requires the influx of extracellular calcium. Increased intracellular calcium leads to the activation of a phospholipase by a Ca^{2+}-calmodulin-mediated pathway. In hormone-stimulated prostaglandin synthesis, the calcium influx appears to be transient, as reflected by the transient release of arachidonic acid. The calcium influx induced by the ionophore A23187 is more sustained, resulting in a longer period of phospholipase stimulation. Other ions, such as manganese, although being transported effectively by A23187, are not active.

ACKNOWLEDGMENTS

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REFERENCES

1. Hassid, A. (1982) Am. J. Physiol. 243, C205-C211.
2. Irvine, R.F. (1982) Biochem. J. 204, 3-16.
3. Van den Bosh, H. (1980) Biochim. Biophys. Acta 604, 191-246.
4. Knapp, H.R., Oelz, O., Roberts, L.J., Sweetman, B.J., Oates, J.A., and Reed, P.W. (1977) Proc. Natl. Acad. Sci. USA 74, 4251-4255.
5. Brotherton, A.F.A., and Hoak, J.C. (1982) Proc. Natl. Acad. Sci. USA 79, 495-499.
6. Juan, H. (1979) Arch. Pharmacol. 307, 177-183.
7. Whorton, A.R., Young, S.L., Data, J.L., Barchowsky, A., and Kent, R.S. (1982) Biochim. Biophys. Acta 712, 79-87.
8. Rittenhouse-Simmons, S. (1981) J. Biol. Chem. 256, 4153-4155.
9. Rittenhouse-Simmons, S. (1979) J. Clin. Invest. 63, 580-587.
10. Rittenhouse-Simmons, S., and Deykin, D. (1978) Biochim. Biophys. Acta 543, 409-422.
11. Hong, S.L., and Deykin, D. (1981) J. Biol. Chem. 256, 5215-5219.
12. Scharschmidt, L., Dunn, M., Ackawa, M., and Hassid, A. (1982) Kidney Int. 21, 265.
13. Wong, P.Y.K., and Cheung, W.Y. (1979) Biochem. Biophys. Res. Commun. 90, 473-480.
14. Jaffe, E.A., Nachman, R.L., Becker, C.G., and Minick, C.R. (1973) J. Clin. Invest. 52, 2745-2756.
15. Whorton, A.R., Carr, K., Smigel, M., Walker, L., Ellis, K., and Oates, J.A. (1979) J. Chromatogr. 163, 64-71.
16. Whorton, A.R., Sweetman, B.J., and Oates, J.A. (1979) Anal. Biochem. 98, 455-463.
17. Weiss, B., Prozialeck, W., Cimino, M., Barnette, M.S., and Wallace, T.L. (1980) Ann. N.Y. Acad. Sci. 356, 319-345.
18. Cheung, W.Y. (1980) Science 207, 19-27.
19. Snedecor, G.W., and Cochran, W.G. (1967) Statistical Methods, 6th edn., University of Iowa Press, Ames.

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The Effect of Dietary Lipid on the Lipoprotein Status of the Mongolian Gerbil[1]

ANDREA B. LEACH and **BRUCE J. HOLUB***, *Department of Nutrition, College of Biological Science, University of Guelph, Guelph, Ontario N1G 2W1, Canada*

ABSTRACT

The Mongolian gerbil, *Meriones unguiculatus*, may be a suitable animal model for the investigation of dietary lipid effects on cholesterol metabolism. The effects of dietary cholesterol, and its possible interaction with the type of dietary fat, on the lipoprotein status of this animal have not been examined previously. In the present research, the effects of adding 0.5% cholesterol to diets high in saturated (19.5% beef tallow: 0.5% safflower oil) or polyunsaturated (20% safflower oil) fats on the lipoprotein status of the gerbil were determined after 11 and 22 days of feeding. Lipoproteins (VLDL, LDL and HDL) were separated by sequential ultracentrifugation. Their cholesterol, phospholipid and protein concentrations were determined colorimetrically. In the absence of 0.5% cholesterol, safflower oil lowered the concentration (mg/100 ml) of cholesterol in each of the VLDL, LDL and HDL relative to beef tallow (BT) without greatly influencing the cholesterol distribution amongst them. The HDL carried the majority of the serum cholesterol and the VLDL transported the smallest amount. However, inclusion of 0.5% dietary cholesterol resulted in a redistribution of cholesterol amongst the lipoproteins so that the VLDL and LDL became the major and the HDL the minor carriers. Dietary cholesterol also brought about a rise in the VLDL and LDL concentrations (mg/100 ml) of cholesterol, phospholipid and protein and altered the VLDL and LDL compositions. No such changes were observed in the HDL, indicating that the HDL are relatively resistant to any of the possible effects of cholesterol feeding measured in this experiment. The specific mechanisms responsible for the changes observed in the lipoprotein status of the gerbil remain to be elucidated.

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INTRODUCTION

The positive correlation between the level of total serum cholesterol and the occurrence of atherosclerosis in humans is well recognized (1,2). It is also well known that human serum cholesterol levels are generally inversely related to the degree of polyunsaturation of dietary fat (3). In addition, the level of dietary cholesterol may influence serum cholesterol levels (4). Because of the limitations of using human subjects, several animal species have been used to determine the mechanisms by which the type and amount of dietary lipid exerts their effects on serum cholesterol levels.

In this regard, the rat is the most commonly used model, although it lacks several qualities advantageous to the study of dietary lipid effects on cholesterol metabolism. For example, the response of its serum cholesterol level to the type of dietary fat is often unlike that seen in humans (5). It is also often necessary to add cholic acid, cholestyramine or propylthiouracil to the diets in order to obtain a significant response to dietary fat type (6). Finally, although cholesterol linoleate is the major cholesteryl ester and cholesteryl arachidonate only a minor form in human serum (7), the opposite pattern occurs in the rat (7). The similarity of the gerbil *(Meriones unguiculatus)* to the human in its plasma lipid profile and in the sensitivity and similarity of the response of its plasma cholesterol level to dietary fat type has indicated its potential suitability as an animal model in cholesterol research (8-10). Because of the importance of lipoproteins in cholesterol metabolism as a whole and also as risk factors in the development of atherosclerosis (1), it is important to know what effect dietary lipid may have on the status of these particles. To date, only two studies of this type have been conducted using the gerbil. Nicolosi et al. (11) examined the relationship of the type of dietary fat to the composition of gerbil lipoproteins. Gordon and Cekleniak (12) studied the changes in the distribution of cholesterol, which occur with cholesterol feeding, amongst the VLDL and LDL. Apparently, the status of the HDL was not measured. The diets used in these studies were not similar to that which is typically consumed by North Americans.

The present study examined the trends occurring in the lipoproteins of Mongolian gerbils on diets similar to that consumed by

[1] Presented in part at the Triennial Joint Meeting of the AIN/ASCN/CSNS, July 1982.

*To whom correspondence should be addressed.

Abbreviations: BT, beef tallow; C, cholesterol; SO, safflower oil; VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; P/S, polyunsaturated/saturated.

TABLE 1

Composition of Experimental Diets

Constituent	Sterol-equalized experimental diets	
	Safflower oil ±0.5% cholesterol	Beef tallow ±0.5% cholesterol
	(% by weight)	
Casein	16	16
Fat		
Beef tallow	–	19.5
Safflower oil	20	0.5[a]
Carbohydrate		
Corn starch	36.3	36.3
Sucrose	18.2	18.2
Alfa floc	4.2	4.2
Salt mixture[b]	4.0	4.0
Vitamin mixture[c]	1.0	1.0
Choline chloride	0.3	0.3
Sterol		
Phytosterol	0.048	0.048[d]
Cholesterol	0.010[d] or 0.510	0.010 or 0.510

[a]Safflower oil (0.5%) was added to the diets containing beef tallow to ensure the presence of adequate amounts of essential fatty acids.

[b]Williams-Briggs Modified, Teklad Mills, Madison, WI.

[c]Amounts in mg/kg diet: DL-α-tocopherol acetate, 160; menadione, 0.2; thiamine-HCl, 6.0; riboflavin, 7.0; pyridoxine-HCl, 7.0; DL-calcium pantothenate, 20.0; nicotinic acid, 25.0; D-biotin, 0.2; folic acid, 0.1; cyanocobalamin, 0.012; ergocalciferol, 4.0; retinyl acetate, 17.45. This mix also includes in mg/kg diet: sodium selenite, 0.22; chromium acetate, 2.3; mesoinositol dihydrate, 1,200; L-methionine, 1,000. The vitamin mix contained 75% alfa floc as carrier. Therefore, the diet contains 5% alfa floc.

[d]These sterols were not endogenous to their respective diets and were therefore added to equalize all diets for their sterol content. Phytosterol was added as β-sitosterol.

North Americans in the levels of protein, fat and carbohydrate, and in the type of carbohydrate (13,14) but in which the effect of cholesterol supplementation was studied with varied P/S ratios in the diet. Thus, it was possible to compare the similarities or differences between dietary cholesterol and dietary fat type in altering the lipoprotein status of this animal. Because sterols present endogenously in the fats could possibly alter serum cholesterol levels (15), all dietary treatments used in the present experiment were equalized for the endogenous sterol content of the fats used.

MATERIALS AND METHODS

Experimental Procedures

Animals and housing conditions. Forty-eight male, adolescent Mongolian gerbils (High Oak Ranch, Goodwood, Ontario), with an average body weight of 38 g, were maintained on Purina Lab Chow 5012. They were housed in pairs in wire-bottomed, stainless-steel cages in a room with a 12-hr light/dark lighting schedule and at a constant temperature of 25 C. After 2 weeks of adaptation, the animals were randomly assigned to one of 4 experimental diets and continued to receive ad libitum access to their food and water. Their body weights and feed intakes were measured every 3 days.

Diets. The compositions of the experimental diets are as shown in Table 1. Sources of protein, fat and carbohydrate were added at levels which would provide a caloric distribution equivalent to that seen in the typical North American diet (13,14). Similarly, the starch: sucrose ratio was also typical of the North American diet (13,14). The two experimental fats were beef tallow (BT) and safflower oil (SO). Four diets were used; two of these contained 20% SO, and the other two contained 19.5% BT + 0.5% SO (added to ensure the presence of adequate levels of essential fatty acids). To each of the SO diets, 0.010% cholesterol (C) was added in order to equalize them with respect to the endogenous cholesterol

TABLE 2

Fatty Acid Composition of Dietary Lipids Used to Determine the Effects of Fat Type and Cholesterol on the Lipoprotein Status of the Male Mongolian Gerbil

Fatty acid	Safflower oil	Safflower oil + cholesterol	Beef tallow	Beef tallow + cholesterol
		(wt % of the total[a])		
14:0	tr	tr	2.3	2.7
16:0	8.1	8.7	27.9	28.7
16:1	tr	tr	5.3	5.4
18:0	2.9	2.6	20.2	19.6
18:1	13.1	12.4	37.3	37.2
18:2	75.8	76.3	5.0	4.9
Total saturated fatty acids	11.0	11.3	50.4	51.0
P/S ratio[b]	6.9	6.7	0.1	0.1

[a]Fatty acids representing less than 1.5% of the total of any dietary lipid have been omitted from the table. (tr = trace amounts, i.e., less than 1.5% of the total fatty acids).

[b]Calculated as total % of polyunsaturated fatty acids divided by total % of saturated fatty acids.

content of BT. Similarly, 0.048% β-sitosterol (Serdary Research Labs, London, Ontario) was added to the BT diets to equalize them for the endogenous phytosterol of SO. These levels of cholesterol and β-sitosterol were chosen because they were the levels of sterol found to be present in BT and SO, respectively, by Mercer (16). Subsequent analysis of the BT and SO for their sterol content showed that they contained 80-84% of the amount of sterol actually added to the diets. Finally, 0.5% cholesterol was added to one of each of the sterol-equalized BT and SO diets. The 4 diets will subsequently be called BT, BT+C, SO and SO+C. The fatty acid compositions of the fats in the 4 diets and their P/S ratios appear in Table 2. The P/S ratio of the SO-containing diets was ca. 6.8, and that of the BT diets was 0.1.

Methods. After 11 and 22 days on the diets, the gerbils were fasted for 12-15 hr. After anesthetization with Metofane (Methoxyflurane, Pitman-Moore, Washington Crossing, NJ), ca. 2 ml of blood were collected from each animal by heart puncture. For each dietary group and sampling period, 6 animals were used. Serum was obtained and that of cagemates was pooled. Twenty-μl aliquots of each pooled serum sample were analyzed for total serum cholesterol content by the colorimetric procedure of Bhandaru et al. (17) using a cholesterol standard dissolved in chloroform. The remaining serum was frozen at −70 C until needed for the separation of lipoproteins.

In order to have an adequate volume for ultracentrifugation, the serum was pooled according to dietary treatment and time of sampling. This left only one sample for each dietary group at each time of sampling. The VLDL, LDL and HDL were separated by a modification of the sequential ultracentrifugation procedure of Lindgren et al. (18) as described by Hatch and Lees (19). The exact volume of each of the lipoprotein fractions collected was measured by Hamilton syringe. Forty-μl aliquots were removed to a test tube for protein analysis by the method of Lowry et al. (20). Preliminary tests had shown that the salt solutions used for ultracentrifugal separation did not cause interference in the Lowry method. The rest of the supernatant was transferred to a tube containing 16 ml of chloroform/methanol (2:1, v/v). The mixture was shaken for 30 sec on a vortex mixer. Ca. 1.7 ml water (2.0 − amount of supernatant added) were added and, after mixing by vortex for 15 sec, the tubes were centrifuged for 5 min at 250 × g.

The lower chloroform phase was removed by pasteur pipette and used for determination of lipoprotein cholesterol by colorimetry (17) and total phospholipid by a modification of the procedure of Bartlett (21) to allow for microdeterminations of inorganic phosphorus (22). For the phosphorus determination, a standard aqueous solution of sodium dihydrogen phosphate was used. Lipid phosphorus was determined using a conversion factor of 25. It should be noted that all glassware used in this analysis must be completely free of phosphorus. This required washing of such apparatus in a hot mixture of concentrated HCl and H_2O (4:1, v/v) for ca. 4 hr.

Statistical analyses. Data, where applicable, were subjected to analysis of variance for a

TABLE 3

Effect of Dietary Lipid on the Total Serum Cholesterol of the Mongolian Gerbil[a]

Diet	Total serum cholesterol (mg/100 ml) Day 11	Day 22
Beef tallow	133 ± 6[a]	100 ± 9[a]
Beef tallow + cholesterol	457 ± 32[b]	425 ± 18[b]
Safflower oil	90 ± 18[c]	52 ± 5[c]
Safflower oil + cholesterol	383 ± 24[d]	542 ± 22[d]

[a]All values are given as mean ± SEM for 3 samples/group. Values in a column not sharing a common letter are significantly different at $p < 0.05$.

2 × 2 factorial experimental design (23). The level of significance was chosen to be $p < 0.05$.

RESULTS

No significant differences were seen among groups with respect to cumulative weight gains, feed intakes or weight gain/feed intake ratios. In general, the recovery of lipoproteins was within ±10% of the total as measured by total serum cholesterol. By day 11, there was a lowering of serum cholesterol caused by dietary safflower oil relative to beef tallow, regardless of the amount of cholesterol in the diets (Table 3). In addition, the BT+C and SO+C diets produced at least a 4-fold increase in total serum cholesterol (Table 3) relative to the BT and SO diets, respectively. By the end of the third week, differences in total serum cholesterol resulting from both cholesterol feeding and from the type of dietary fat were still apparent. At this time of sampling, when there was little cholesterol in the diets, safflower oil lowered the serum cholesterol compared to beef tallow but, with the inclusion of 0.5% cholesterol, the opposite trend was seen (Table 3).

Figure 1 clearly demonstrates the dramatic rise in concentration (mg/100 ml or mg percent) of VLDL and LDL cholesterol, at both 11 and 22 days, that is brought about by 0.5% dietary cholesterol. Contrary to this, the HDL appear to be resistant to any changes, not only in their cholesterol content, but also in their phospholipid and protein levels (Figs. 2 and 3). However, the latter two components were markedly higher in the VLDL and LDL of animals fed 0.5% cholesterol vs those that were not given cholesterol (Figs. 2 and 3).

The cholesterol concentration of the individual lipoproteins was also influenced by dietary fat type (Fig. 1). When exogenous cholesterol was not included in the diets, the SO tended to produce lower concentrations of cholesterol in the VLDL, LDL and HDL. However, when 0.5% cholesterol was added, no consistent changes due to dietary fat type were observed

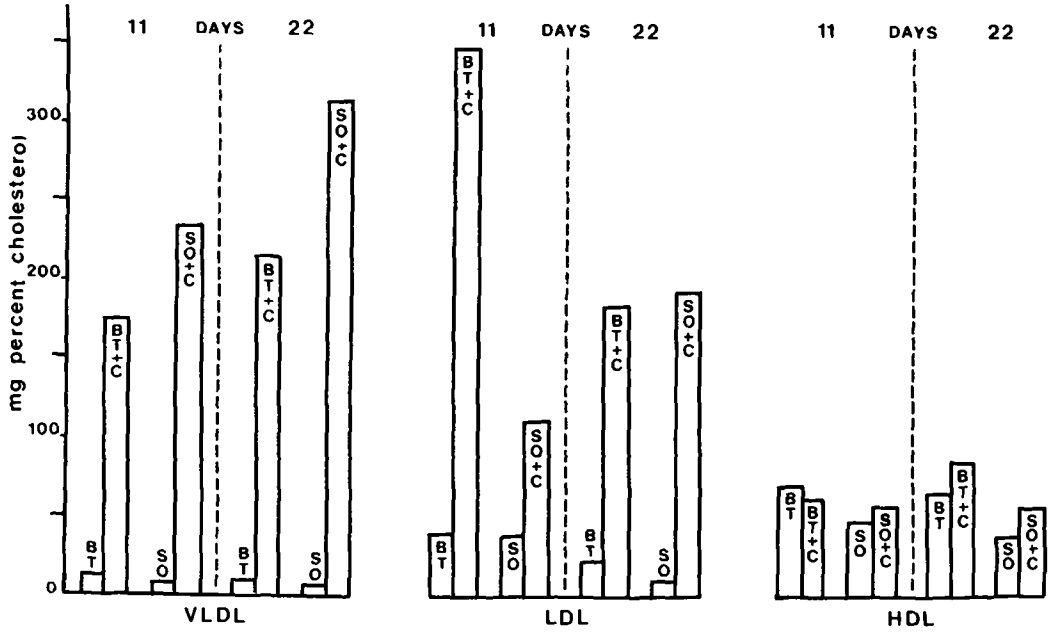

FIG. 1. The response of the cholesterol concentration of each of the VLDL, LDL and HDL in the gerbil after 11 or 22 days of cholesterol feeding. BT = beef tallow; BT+C = beef tallow + cholesterol; SO = safflower oil; SO+C = safflower oil + cholesterol.

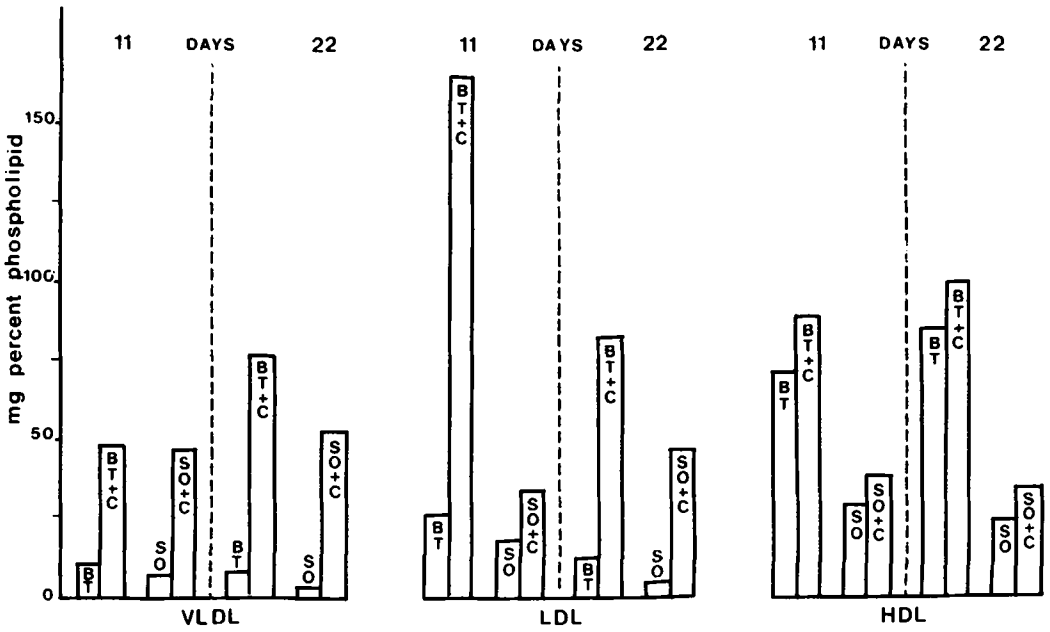

FIG. 2. The response of the phospholipid concentration of each of the VLDL, LDL and HDL in the gerbil after 11 or 22 days of cholesterol feeding. BT = beef tallow; BT+C = beef tallow + cholesterol; SO = safflower oil; SO+C = safflower oil + cholesterol.

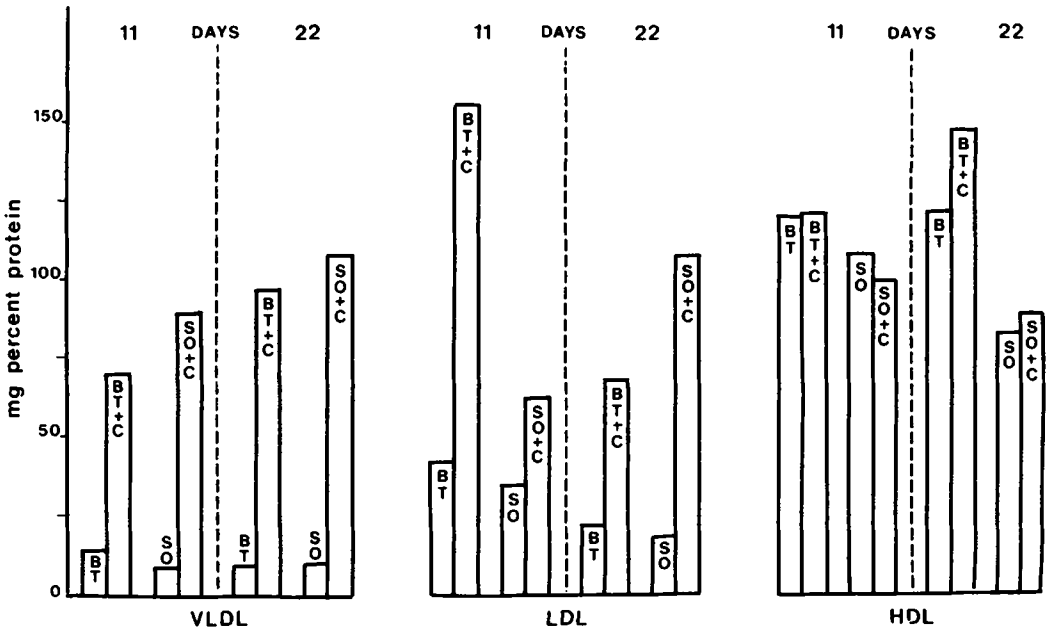

FIG. 3. The response of the protein concentration of each of the VLDL, LDL and HDL in the gerbil after 11 or 22 days of cholesterol feeding. BT = beef tallow; BT+C = beef tallow + cholesterol; SO = safflower oil; SO+C = safflower oil + cholesterol.

TABLE 4

Effect of Cholesterol Feeding on the Protein Concentration and the Relative Amounts of Cholesterol, Phospholipid and Protein in the VLDL

Diet	Days on diet	Cholesterol / Phospholipid	Cholesterol / Protein (wt/wt)	Phospholipid / Protein	Protein (mg/100 ml serum)
Beef tallow	11	1.2	0.9	0.7	14
Safflower oil	11	1.3	0.8	0.6	9
Beef tallow	22	1.2	1.1	0.9	9
Safflower oil	22	1.7	0.3	0.2	10.5
Beef tallow + cholesterol	11	3.6	2.5	0.7	70
Safflower oil + cholesterol	11	5.1	2.7	0.5	87
Beef tallow + cholesterol	22	2.8	2.3	0.8	96
Safflower oil + cholesterol	22	6.0	3.0	0.5	107

in the cholesterol concentration of the three lipoproteins (Fig. 1). It is also apparent from Figure 1 that, in gerbils consuming diets containing either BT or SO, the majority of the total cholesterol is contained within the HDL and the VLDL are the minor carriers of cholesterol. This distribution changes markedly with 0.5% cholesterol feeding. In the latter case, regardless of dietary fat type, the VLDL and LDL now carry the majority of the total serum cholesterol and the HDL transport the least amount. This difference is reflected in the HDL/LDL cholesterol ratios. The ratio in animals fed cholesterol is ca. 1/8 of that found in the animals on the low-cholesterol diets.

The cholesterol:phospholipid, cholesterol: protein and phospholipid:protein ratios (on a weight/weight basis) were determined for each lipoprotein in order to assess whether 0.5% dietary cholesterol produced any compositional changes in the particles. These ratios for the VLDL, LDL and HDL are depicted in Tables 4, 5 and 6, respectively. Protein concentrations are also included in each table to serve as baseline indicators for changes in concentration of the lipoproteins. In the VLDL, cholesterol feeding brought about elevations in the first two of the ratios examined and higher protein concentrations (Table 4). In the LDL, only the cholesterol:protein ratio was markedly raised by dietary cholesterol; the protein concentration was also higher (Table 5). Finally, it can be clearly seen from Table 6 that cholesterol feeding produced very little change in any of the ratios or in the protein concentration of the HDL.

DISCUSSION

In the present study, the gerbils' serum cholesterol levels were lowered by SO relative to BT after 11 days, regardless of the cholesterol content of the diets. This lowering effect of serum cholesterol by a polyunsaturated fat relative to a saturated fat agrees with the reports of Mercer and Holub (8), Hegsted and Gallagher (10) and Nicolosi et al. (11). The sensitivity of the response is in contrast to that seen in the rat and indicates the greater usefulness of the gerbil as an animal model. On day 22, this lowering effect of SO was only seen in the serum cholesterol of animals consuming diets low in cholesterol. The effect of fat type was reversed on day 22 when 0.5% cholesterol was included in the diets. Further work would be necessary to determine if the gerbils may be exhibiting a somewhat unique adaptive effect to diets containing SO plus cholesterol.

The serum cholesterol level of the gerbil is also sensitive to dietary cholesterol, as evidenced by the rise (at least 4-fold) in cholesterol levels seen in Table 3. Although the levels reached may appear to be much higher than normally observed in humans, it should be pointed out that 0.5% cholesterol represents a human intake of ca. 2.5 g of cholesterol/day. This value was calculated from the assumed consumption of ca. 2,500 kcal/day by humans (24) with 1 g of the gerbils' diets supplying ca. 5 kcal (16). The 2.5 g of cholesterol/day is substantially above the 450-600 mg cholesterol normally ingested per day (13,14,25). However, using 0.1% dietary cholesterol (which is ap-

TABLE 5

Effect of Cholesterol Feeding on the Protein Concentration and the Relative Amounts of Cholesterol, Phospholipid and Protein in the LDL

Diet	Days on diet	Cholesterol / Phospholipid	Cholesterol / Protein (wt/wt)	Phospholipid / Protein	Protein (mg/100 ml serum)
Beef tallow	11	1.4	1.0	0.6	43
Safflower oil	11	2.2	1.0	0.5	36
Beef tallow	22	2.0	1.0	0.5	22
Safflower oil	22	2.5	0.6	0.3	17
Beef tallow + cholesterol	11	2.1	2.2	1.1	156
Safflower oil + cholesterol	11	3.3	1.8	0.6	62
Beef tallow + cholesterol	22	2.2	2.6	1.2	70
Safflower oil + cholesterol	22	4.2	1.8	0.4	107

TABLE 6

Effect of Cholesterol Feeding on the Protein Concentration and the Relative Amounts of Cholesterol, Phospholipid and Protein in the HDL

Diet	Days on diet	Cholesterol / Phospholipid	Cholesterol / Protein (wt/wt)	Phospholipid / Protein	Protein (mg/100 ml serum)
Beef tallow	11	1.0	0.5	0.6	120
Safflower oil	11	1.6	0.4	0.3	107
Beef tallow	22	0.8	0.5	0.7	121
Safflower oil	22	1.6	0.5	0.3	83
Beef tallow + cholesterol	11	0.7	0.5	0.7	121
Safflower oil + cholesterol	11	1.5	0.6	0.4	100
Beef tallow + cholesterol	22	0.9	0.6	0.7	147
Safflower oil + cholesterol	22	1.6	0.6	0.4	89

proximately equivalent to 500 mg cholesterol/day), Andersen and Holub (26) reported plasma cholesterol levels of 179-227 mg/100 ml plasma in the gerbil and these are within the normal range of plasma cholesterol levels in the human (27).

The effects of dietary fat type and cholesterol on the lipoprotein status appear to differ. The lowering of the cholesterol concentrations (mg/100 ml serum) of all 3 lipoproteins by SO vs BT in the absence of 0.5% cholesterol, with little change in the percent distribution of cholesterol amongst the lipoproteins, is similar to the observations of Nicolosi et al. (11). The lack of a general lowering of the mg cholesterol/100 ml of serum in the VLDL and LDL by the SO+C diet relative to the BT+C diet indicates that the great increase in the cholesterol load of these lipoproteins, resulting from the 0.5% dietary cholesterol, masks any effect of dietary fat type on the cholesterol content.

When fed a low cholesterol (ca. 0.01%) diet, the gerbil differs somewhat from the human in the distribution of its cholesterol among the lipoproteins. The distribution in the animals consuming both the BT or SO diets concur with

the distributions found by Nicolosi et al. (11) and Mercer et al. (28). However, as suggested previously, ca. 0.1% of the human diet consists of cholesterol. When the gerbils were given 0.5% dietary cholesterol in the present study, the cholesterol distribution changed markedly so that the VLDL and LDL became the major carriers of cholesterol and the HDL the minor ones. This agrees with the results of Gordon and Cekleniak (12), who analyzed the cholesterol content of the lipoproteins after 3 weeks of feeding 1% cholesterol. Thus, it could be speculated that if gerbils were placed on a diet containing ca. 0.1% cholesterol, their lipoproteins might exhibit a cholesterol distribution which would be closer to that seen normally in the human. It is interesting that the pattern of cholesterol distribution found in the gerbils on the low cholesterol diets agrees generally with that seen in the rat, mouse and hamster (29).

Prior to the present study, little was known about the influence of dietary cholesterol on the HDL status of the gerbil. The lack of response of HDL concentration and composition to cholesterol feeding that was observed is not an uncommon finding in the few human studies conducted so far. In the work of Applebaum-Bowden et al. (30) and O'Brien and Reiser (31), total plasma cholesterol was elevated with cholesterol feeding but HDL cholesterol did not change significantly. Flaim et al. (32) and Stein et al. (33) observed no changes in total or HDL cholesterol when their subjects' cholesterol intake varied from 160 to 450 mg/day and 400 to 1400 mg/day, respectively. Perhaps the most substantial evidence comes from the work of Schonfeld et al. (34) who found no significant change in HDL cholesterol levels when male subjects were given diets of P/S ratio 0.25 and 650-1500 mg cholesterol/day or of P/S ratio 0.8 and 1500 mg cholesterol/day, even though total cholesterol was significantly above baseline. Tan et al. (35) reported a 30% rise in HDL cholesterol, but this was caused by a diet in which both the cholesterol level and the P/S ratio had been altered, and Schaefer et al. (36) reported no decrease in HDL cholesterol due to reduced cholesterol consumption.

The results obtained for the status of the HDL do not eliminate the possibility of a change in the size of the particles. The cholesterol-fed gerbils could have either larger but fewer particles or more particles of a smaller size relative to those of the animals on the low cholesterol diets. Furthermore, there may have been alterations in the composition of the HDL subfractions. It is well known that β-VLDL and HDL lipoproteins become induced in most cholesterol-fed animals (37) and this may be the case in humans (38,39). In addition, the HDL_2/HDL_3 ratio can be altered in the human by dietary cholesterol even if total HDL cholesterol is not (34). Therefore, further investigation into the gerbil HDL subfraction composition is warranted.

It is understandable that cholesterol feeding resulted in very little alteration in the HDL composition or of their concentrations of protein, phospholipid and cholesterol when it is considered that, in the pathway of lipoproteins metabolism, the HDL are the farthest removed of all the lipoproteins from the effects of dietary variations. In the cholesterol-fed animals, the greatest percentage of total plasma cholesterol was contained within the VLDL and LDL. This might be expected since it is within these particles that dietary cholesterol received by the liver is packaged and then transported to the peripheral cells. The higher levels of mg protein/100 ml serum found in the VLDL and LDL with cholesterol feeding could indicate a rise in the synthetic rate of the particles leading to increased numbers of particles. Frnka and Reiser (40) found that, in the rat, altered levels of serum lipoproteins, produced by dietary cholesterol, arise mainly from altered rates of synthesis of apoprotein moieties.

The VLDL have also undergone compositional changes, presumably to accommodate increased levels of cholesterol in the particles. A change in composition is also apparent in the LDL, which are the breakdown products of the VLDL. It is doubtful whether the peripheral cells would have increased their uptake of LDL particles since their cholesterol requirements should not have changed. Therefore, with continued intake of high levels of cholesterol, one might expect a greater accumulation of cholesterol within the LDL. This is borne out in the study of Gordon and Cekleniak (12), who found that the LDL became the major carriers of cholesterol after long-term feeding of cholesterol. A similar rate of uptake of LDL by peripheral cells in both control and cholesterol-fed groups in the present experiment should also produce a similar rate of removal of cholesterol from the tissues. If the HDL of the gerbil are used for this purpose, it is then apparent why the HDL were resistant to any changes. Clearly, there is a need for metabolic studies in order to determine the real basis for the phenomena seen in this study.

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REFERENCES

1. Gordon, T., Castelli, W.P., Hjortland, M.E., Kannel, W.B., and Dawber, T.R. (1977) J. Am. Med. Assoc. 238, 497-504.
2. Keys, A. (1972) in Preventive Cardiology (Tiblin, G., Keys, A., and Werkö, L., eds.) pp. 21-34, Almqvist and Wiksell, Stockholm.
3. Keys, A., Anderson, J.T., and Grande, F. (1957) Lancet 273, 959-966.
4. Connor, W.E. (1968) J. Am. Diet. Assoc. 52, 202-208.
5. Hegsted, D.M., Gotsis, A., and Stare, F.J. (1957) J. Nutr. 63, 377-391.
6. Dolphin, P.J. (1981) J. Lipid Res. 22, 971-989.
7. Swell, L., Field, H., Jr., and Treadwell, C.R. (1960) Proc. Soc. Exp. Biol. Med. 104, 325-328.
8. Mercer, N.J.H., and Holub, B.J. (1979) Lipids 14, 1009-1014.
9. Albers, H.J., and Gordon, S. (1962) Proc. Soc. Exp. Biol. Med. 109, 860-863.
10. Hegsted, D.M., and Gallagher, A. (1967) J. Lipid Res. 8, 210-214.
11. Nicolosi, R.J., Marlett, J.A., Morello, A.M., Flanagan, S.A., and Hegsted, D.M. (1981) Atherosclerosis 38, 359-371.
12. Gordon, S., and Cekleniak, W.P. (1961) Am. J. Physiol. 201, 27-28.
13. Committee on Diet and Cardiovascular Disease (1976) Report to the Honourable Marc Lalonde, Minister of National Health and Welfare, pp. 145-147, Bureau of Nutritional Sciences, Health Protection Branch, Department of National Health and Welfare.
14. Select Committee on Nutrition and Human Needs, U.S. Senate (1977) Dietary Goals for the United States, pp. 12-15, U.S. Government Printing Office.
15. Beveridge, J.M.R., Huast, H.L., and Connell, W.P. (1964) J. Nutr. 83, 119-122.
16. Mercer, N.J.H. (1980) Ph.D. Thesis, University of Guelph, Ontario.
17. Bhandaru, R.R., Srinivasan, S.R., Pargaonkar, P.S., and Berenson, G.S. (1977) Lipids 12, 1078-1080.
18. Lindgren, F.T., Nichols, A.V., and Wills, R.D. (1961) Am. J. Clin. Nutr. 9, 13-23.
19. Hatch, F.T., and Lees, R.S. (1968) Adv. Lipid Res. 6, 2-68.
20. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
21. Bartlett, G.R. (1959) J. Biol. Chem. 234, 466-468.
22. Marinetti, G.V. (1961) J. Lipid Res. 3, 1-20.
23. Snedecor, G.W., and Cochran, W.G. (1980) in Statistical Methods, 7th edn., pp. 252-254, The Iowa State University Press, Ames, IA.
24. Passmore, R., Nicol, B.M., Rao, M.N., Beaton, G.H., and De Maeyer, E.M. (1974) in Handbook on Human Nutritional Requirements, FAO Nutritional Studies no. 28, Rome.
25. Levy, R.I. (1981) Clin. Chem. 27, 653-662.
26. Andersen, D.B., and Holub, B.J. (1982) Can. J. Physiol. Pharmacol. 60, 885-892.
27. Hewitt, D., Jones, G.J.L., Godin, G.J., McComb, K., Breckenridge, W.C., Little, J.A., Steiner, G., Mishkel, M.A., Baillie, J.H., Martin, R.H., Gibson, E.S., Prendergast, W.F., and Parliament, W.J. (1977) Can. Med. Assoc. J. 117, 1020-1024.
28. Mercer, N.J.H., Campbell, H., and Carroll, K.K. (1982) J. Nutr. 120, xxv, 61.
29. Tsuda, M., Kitazaki, T., and Imai, Y. (1983) J. Biochem. 93, 1071-1077.
30. Applebaum-Bowden, D., Hazzrd, W.R., Cain, J., Cheung, M.C., Kushwaha, R.S., and Albers, J.J. (1979) Atherosclerosis 33, 385-396.
31. O'Brien, B.C., and Reiser, R. (1980) Am. J. Clin. Nutr. 33, 2573-2580.
32. Flaim, E., Ferreri, L.F., Forrest, W.T., Hill, J.E., and Ritchey, S.J. (1981) Am. J. Clin. Nutr. 34, 1103-1108.
33. Stein, E.A., Shapero, J., McNerney, C., Glueck, C.J., Tracy, T., and Gartside, P. (1982) Am. J. Clin. Nutr. 35, 1375-1390.
34. Schonfeld, G., Patsch, W., Rudel, L.L., Nelson, C., Epstein, M., and Olson, R.E. (1982) J. Clin. Invest. 69, 1072-1080.
35. Tan, W.H., Dickinson, M.A., Albers, J.J., Havel, R.J., Cheung, M.C., and Vigne, J.L. (1980) Am. J. Clin. Nutr. 33, 2559-2565.
36. Schaefer, E.J., Eisenberg, S., and Levy, R.I. (1978) J. Lipid Res. 19, 667-687.
37. Mahley, R.W. (1978) in Disturbances in Lipid and Lipoprotein Metabolism (Dietschy, J.M., Gotto, A.M., Jr., and Ontko, J.A., eds.) pp. 181-197, American Physiological Society, Bethseda, MD.
38. Mistry, P., Nicholl, A., Nichaus, C., Christie, I., Janus, E., and Lewis, B. (1976) Circulation 54, II-78.
39. Mahley, R.W., Innerarity, T.L., Bersot, T.P., Lipson, A., and Margolis, S. (1978) Lancet 2, 807-809.
40. Frnka, J., and Reiser, R. (1974) Biochim. Biophys. Acta 360, 322-338.

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Lipoxygenase Inhibition by Naturally Occurring Monoenoic Fatty Acids

A.J. ST. ANGELO and **R.L. ORY**, *Southern Regional Research Center, PO Box 19687, New Orleans, LA 70179*

ABSTRACT

Lipoxygenase activity was not detected in preparations from several varieties of rapeseed. Erucic acid, one of the main components of rapeseed oil, competitively inhibited both soybean and peanut lipoxygenases. Various other long-chain monoenoic fatty acids were assayed for their effects on lipoxygenase activity with linoleic acid as substrate. Fatty acid chain length, from C_{16} to C_{24}, was not a significant factor, but position of the point of unsaturation did affect enzyme activity. The position of the double bond from the carboxyl group seemed to be more significant than the distance between the point of unsaturation and the methyl terminal group in terms of inhibition.
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Lipoxygenase (EC 1.13.1.13) activity has been reported in most plant sources, such as fruits, vegetables and oilseeds (1,2). During the past decade, lipoxygenase or lipoxygenase-like enzymes have been found in animal tissues (3). Among the oilseeds, the most active source of the enzyme is the soybean. Rapeseed, which ranks fifth in abundance among oilseeds of the world, appears to be an exception. Franke and Frehse (4) reported no activity in rapeseed compared to 9 oilseeds high in triglycerides of linoleic/linolenic acids. Bronisz et al. (5) reported only slight activity in rapeseed extracts at pH 6.0-6.5 compared to that in soybean. Appleqvist (6) believes that lipoxygenase activity exists in dormant rapeseed but theorized that it was not detected because of the possible presence of inhibitor(s) in the seed.

Since erucic acid is the most abundant fatty acid in rapeseed (up to 40%) and erucic acid inhibits fatty acid oxidation in mitochondria from rat heart and liver (7), St. Angelo et al. (8) tested erucic acid with soybean and peanut lipoxygenase systems and found that small amounts of erucic acid completely inhibited both lipoxygenases. Erucic acid is a 22-carbon *cis*-monounsaturated fatty acid, with unsaturation at the C_{13} position. Because erucic acid inhibited soybean and peanut lipoxygenases, the rapeseed enzyme might be naturally inhibited by erucic acid in the seed. Erucic acid concentration varies among the different rapeseed varieties, so that several varieties were examined for possible lipoxygenase activity. Whether the mechanism of inhibition is caused by position of the double bond (relative to the carboxyl or terminal end groups) or by the carbon chain length is unknown. Hammerstrom (9) selectively inhibited *n*-8 lipoxygenase but not fatty acid cyclooxygenase in human platelets with 5,8,11-eicosatriynoic acid. To determine this mechanism of inhibition and to seek naturally occurring inhibitors, several naturally occurring monoenoic *cis*-fatty acids were examined for their effects on activity of peanut and soybean lipoxygenase.

MATERIALS AND METHODS

Seeds and Materials

Fatty acids (oleic, *cis*-vaccenic, *cis*-11-eicosenoic, *cis*-5-eicosenoic, erucic, nervonic, linoleic) were purchased from Applied Science Laboratories Inc., State College, PA. Virginia 56R peanuts (1972 crop) were certified seed purchased from a Virginia supplier. Rapeseed (*Brassica napus*) samples were: Panter, high erucic acid (54%), a gift from Dr. R. Ohlson, AB Karlshamns Oljefabriker, Sweden; Oro, Low erucic acid (1.1%), Zephyr (0.2%), and a low glycosinolate (0.7%) variety, Bronowski (12-15% erucic acid), were gifts from Dr. W. Calhoun, Oregon State University. Soybean lipoxygenase, petroselinic acid and ricinoleic acid were purchased from Sigma Chemical Company, St. Louis, MO.

Peanut lipoxygenase was prepared by homogenizing the testae-free seeds in deionized water (1:10, wt/vol) and centrifuging twice at 12,000 × g to obtain an S-2 supernatant fraction. The S-2 fraction served as the source of enzyme, as described earlier (10).

Rapeseed extracts were prepared by 3 methods. In method 1, 1 g of kernels from high erucic acid seeds (PAN) was homogenized in deionized water (1:10, wt/vol) with a mortar and pestle, then centrifuged at 25,000 × g for

TABLE 1

Effect of Fatty Acid Inhibition Relative to Position of the Point of Unsaturation

Fatty acid	Inhibition (%) 2.5	5.0	10	20 (μmoles)	Chain length	Number of carbons from double bond to: Terminal	Carboxyl
Oleic	48	66	77	89	18	9	9
Ricinoleic	17	35	67	90	18	9	9
Petroselinic	25	44	67	84	18	12	6
Vaccenic (*cis*)	40	70	83	92	18	7	11
5-*cis*-Eicosenoic	0	1	4	37	20	15	5
11-*cis*-Eicosenoic	25	40	62	81	20	9	11
Erucic	21	40	66	88	22	9	13
Nervonic	68	74	83	91	24	9	15

30 min to collect a precipitate, a supernatant and a fat pad. The hulls were similarly extracted in water to obtain an extract for testing as a possible lipoxygenase inhibitor. In method 2, whole rapeseeds with hulls were homogenized in water in the same manner described for whole peanuts. In method 3, whole rapeseeds were first deoiled in acetone or spectral grade hexane with a mortar and pestle, centrifuged and reextracted with hexane to yield a defatted meal, which was extracted with water as above, to obtain the 2 precipitates and supernatants.

Lipoxygenase Assay

Peanut and rapeseed lipoxygenase were assayed spectrophotometrically at 234 nm, which measures the increase in absorption caused by formation of conjugated diene hydroperoxides from linoleic acid at pH 6.2 for peanut (10) and pH 6.2, 7.9 and 9.2 for rapeseed. Soybean lipoxygenase activity was assayed polarographically, by measuring oxygen uptake as described previously (8). The reaction medium contained 20 μg of soybean lipoxygenase, 0.2 M sodium borate buffer, pH 9.0, in a total volume of 2.0 ml. After incubating at room temperature (25 C) for 5 min, 1.0 ml of substrate (0.5 μmol) was added to initiate the reaction. For inhibitor tests, monoenoic fatty acids were dissolved in ethanol, then incubated with the enzyme for 5 min prior to addition of substrate. In controls, a corresponding volume of alcohol was incubated with the enzyme-buffer solution prior to addition of substrate. All reactions were run in triplicate. Activity is reported as the change in oxygen (nmol) consumed/min/3 ml of solution. Linoleic acid was prepared according to Ben-Aziz et al. (11). Kinetic analyses were conducted to determine the apparent K_M and V_{max} by the Lineweaver-Burk plot. Data were plotted by the least squares method with a Heathkit Z-89 computer and a Diablo 630 printer.

RESULTS AND DISCUSSION

Lipoxygenase activity could not be detected in supernatant or precipitate fractions from all 4 varieties of rapeseed. Ammonium sulfate precipitation (50%) and concentration of the four S-2 supernatants also failed to produce any activity. Extracts of the hulls from these 4 varieties did not inhibit an active peanut lipoxygenase, indicating that an inhibitor in the hulls is not the reason for the lack of lipoxygenase activity in rapeseed.

Because erucic acid completely inhibited the action of both soybean and peanut lipoxygenase prepared by methods 1 and 2 (8) on linoleic acid, several other naturally occurring monoenoic *cis*-fatty acids, C_{18-24}, were examined as possible inhibitors to determine any effects of position of the double bond and/or chain length. The results are shown in Table 1 with the percentage inhibition at 4 concentrations, the chain length and the positions of the double bond relative to the carboxyl and terminal methyl groups in the molecule. The fatty acids were dissolved in ethanol since this had not inhibited the reaction at the volumes (10-13 μl) used before (8). At 2.5 and 5.0 μmol, nervonic acid, which has the largest number of carbon atoms between the double bond and the carboxyl group, was the strongest inhibitor, followed by oleic and vaccenic acids. Except for 5-*cis*-eicosenoic acid, all of the other acids tested showed inhibition greater than 62% at the 10 μmol level. At this concentration however, the ethanol (25 μl) caused a 5% loss in enzymatic activity of the control (enzyme, substrate and buffer) and at 20 μmol, the ethanol content (ca. 50 μl) caused 18% inhibition of the control reaction. No

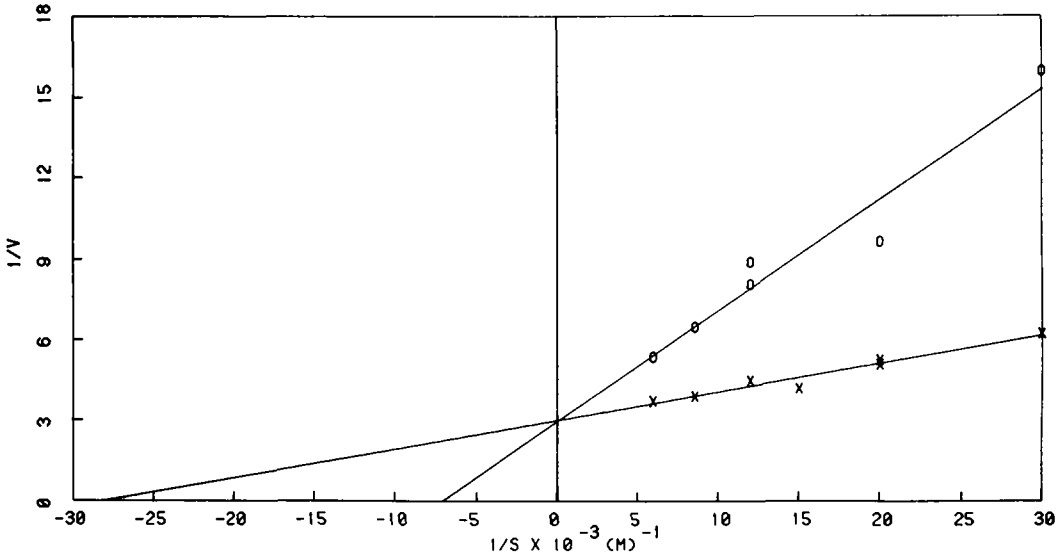

FIG. 1. Lineweaver-Burk plot showing the effect of erucic acid on the standard lipoxygenase-linoleic acid system. Reaction mixture contained 0.05 M phosphate buffer, pH 8.36, soybean lipoxygenase, 0.02 mg, and linoleic acid (x – x) in a total volume of 3.0 ml. Erucic acid, 4.1 mg in 5 μl ethanol, was added in each substrate concentration (O – O). Reaction rates were determined by oxygen electrode as described in Methods.

attempt was made to reverse the inhibitory effect of the fatty acids.

At 2.5 μmol (Table 1), oleic acid is 3 times as inhibitory as ricinoleic acid. At 5.0 μmol, the inhibition by ricinoleic acid doubled and approached that of oleic acid at 10 μmol.

Comparing *n*-9 fatty acids (oleic, C_{18}, 11-eicosenoic, C_{20}, erucic acids, C_{22}) at the 2.5 μmol level, the inhibition was 48%, 25% and 21%, respectively. These results suggest that the distance between the double bond and the carboxyl group and inhibition are not related when the number of carbon atoms is at least 9. Two of the acids tested, vaccenic and 11-eicosenoic, have the same number of carbons between the double bond and the carboxyl group. At both the 2.5 and 5.0 μmol levels, the shorter chain length acid, vaccenic, was the stronger inhibitor.

Enzyme inhibition by erucic acid was found to be the competitive type, based on the Lineweaver-Burk plot (Fig. 1). The apparent K_m with linoleic acid was 3.6×10^{-5} M, whereas the V_{max} was still 2.98. Similar kinetic studies for the other fatty acids that inhibit lipoxygenase were not determined, but one can assume that they are of the same type as erucic acid.

Laakso and Lilius (12) recently described the inhibition of soybean lipoxygenase by casein and alluded to the possibility of spontaneous coupling of casein to the enzyme due to the hydrophobic nature of lipoxygenase. Our data on 5-*cis*-eicosenoic and nervonic acids suggest that nervonic may be complexing with the enzyme through the long hydrophobic region between the carboxyl group and the double bond, whereas 5-eicosenoic acid does not inhibit because of a lack of any appreciable hydrophobic area between those groups. In studies on the protective effect of saturated fatty acids on rate of autoxidation of linoleic acid, Wu et al. (13) showed that maximum effective protection against autoxidation occurs when the methyl terminal end of the saturated acid extends to the vicinity of the methylene-interrupted diene. The acids of medium chain length, C_{10-14}, were the most effective protectors of autoxidation.

REFERENCES

1. Rhee, K.S., and Watts, B.M. (1966) J. Food Sci. 31, 664-668.
2. Pinsky, A., Grossman, S., and Trop, M. (1971) J. Food Sci. 36, 571-572.
3. Gardner, H.W. (1980) in Autoxidation in Food and Biological Systems (Simic, M.G., and Karel, M., eds.) pp. 447-504, Plenum Press, New York, N.Y.
4. Franke, W., and Frehse, H. (1954) Hoppe-Seyler's Z. Physiol. Chemie 298, 1-26.
5. Bronisz, H., Arbatowska, M., and Leszczynska, H. (1958) Chem. Abstr. 52, 13020d; (1958), Roczniki Panstwoweg. Zak. Hig. 9, 29-40.
6. Appleqvist, L.A. (1972) in Rapeseed (Appleqvist, A.A., and Ohlson, R., eds.) p. 164, Elsevier

Publishing Co., Amsterdam, The Netherlands.
7. Christophersen, B.O., and Bremer, H. (1972) Biochim. Biophys. Acta 280, 506-514.
8. St. Angelo, A.J., Kuck, J.C., and Ory, R.L. (1979) J. Agric. Food Chem. 27, 229-234.
9. Hammerstrom, S. (1977) Biochim. Biophys. Acta 487, 517-519.
10. St. Angelo, A.J., and Ory, R.L. (1972) in Symposium: Seed Proteins (Inglett, G.E., ed.) pp. 284-291, Avi Publishing Co., Westport, CT.
11. Ben-Aziz, A., Grossman, S., Ascarelli, I., and Budowski, P. (1970) Anal. Biochem. 34, 88-100.
12. Laakso, S., and Lilius, E.M. (1982) J. Agric. Food Chem. 30, 913-916.
13. Wu, G.S., Stein, R.A., and Mead, J.F. (1978) Lipids 13, 517-524.

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Lung Surfactant and Fatty Acid Composition of Lung Tissue and Lavage of Rats Fed Diets Containing Different Lipids

SYED Q. ALAM* and **BASSIMA S. ALAM**, *Department of Biochemistry, Louisiana State University Medical Center, 1100 Florida Avenue, New Orleans, LA 70119*

ABSTRACT

Two nutritional models, essential fatty acid (EFA) deficiency and the feeding of saturated vs unsaturated fats, were used to determine the effects of dietary lipids on the fatty acid composition of rat lung and lavage. Semipurified diets containing 7% corn oil, 7% hydrogenated coconut oil (EFA-deficient), 10% butter or 10% safflower oil were fed to dams during lactation and thereafter to their offspring for a total of 24 weeks. Lipids were extracted from the lung lavage and lung tissue and their fatty acid composition was determined. The content of dipalmitoylphosphatidylcholine (DPPC), the main surfactant in the lungs, was also determined. The results show that the levels of DPPC in the lungs of rats fed 10% butter decreased although the decrease in the EFA-deficient rats was greater. Comparing rats fed butter with those fed corn oil, there were also modifications in the fatty acid composition of the total lipids and phospholipids of lung tissue and lavage as well as in phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol + phosphatidylserine fractions isolated from the lung tissue. The changes in fatty acid composition were somewhat fewer in rats fed butter then in those fed an EFA-deficient diet. The results suggest that a marginal EFA deficiency produced in rats by long-term feeding of 10% butter may account for the reduction in DPPC levels and in the changes in fatty acid composition in the lung tissue and lavage.
Lipids 19:38-43, 1984.

INTRODUCTION

The alteration of lipid composition of animal tissues by hormonal and nutritional factors has been well established. Among the nutritional factors, the type of the dietary fat present has a profound influence on tissue lipid composition. Lung lipids are also influenced by dietary modifications. The contents of dipalmitoylphosphatidylcholine (DPPC), the main surfactant in the alveoli, are known to be altered by different dietary states (1-3). Food restriction (1) and essential fatty acid (EFA) deficiency (2) can cause a significant decrease in DPPC content in the lung tissue and lavage. The effects of nutritional deprivation on lung phospholipids seem to depend, among other factors, on the stage of development of the animal (4).

The purpose of our investigation was to study the effects of feeding saturated vs unsaturated fats on the fatty acid composition of lung and lavage lipids and on the levels of DPPC in the lung. For comparison, an EFA-deficiency model was also included in this study. To maximize the changes in tissue lipid composition, the feeding of various dietary regimes was initiated at birth and continued for 24 weeks.

*To whom correspondence should be addressed.

MATERIALS AND METHODS

Materials

All organic solvents were of analytical reagent grade and were glass-redistilled before use. The dietary ingredients, including hydrogenated coconut oil (HCO), were purchased from ICN (Cleveland, OH). Corn oil, butter and safflower oil were purchased from a local supermarket. Standards of phospholipids and methyl esters of fatty acids were purchased from Applied Science Inc. (college Park, PA).

Animal Study

Fifteen-day pregnant, Sprague-Dawley rats were fed a stock diet until their pups were delivered. Within 24 hr after delivery, the pups were randomized so that each litter contained 8-9 pups. Throughout lactation the dams were fed semipurified diets containing 7% corn oil (control), 7% HCO (EFA-deficient), 10% butter (low in polyunsaturated fatty acids, high in saturated fatty acids) or 10% safflower oil (high in polyunsaturated fatty acids). The basal diet was the same (AIN-76) in each case (5). It consisted of 20% casein, 63.8% sucrose in groups fed corn oil or HCO; 60.8% sucrose in the remaining groups; 4% cellulose; 10% fat or 7% oil; 4% mineral mixture; 1% vitamin mixture and 0.2% choline chloride. Butylated hydroxytoluene (BHT) was added at a level of 0.02% as an antioxident in each of the

4 diets. Diets were freshly prepared every 2-3 weeks and were stored at refrigeration temperature. The pups were weaned at 3 weeks, maintained in individual wire-bottomed, galvanized cages and fed ad libitum the same diets previously fed to their mothers. Weight gains were measured at regular intervals.

Twenty-four weeks after the nutritional experiment began (3 weeks of lactation and 21 weeks after weaning), 4 male rats from each dietary group were anesthetized with sodium pentobarbital and their lungs were lavaged through an endotracheal tube using 5 x 5 ml of 0.05 M Tris/0.15 M NaCl with a pH of 7.4. The lungs were quickly dissected, weighed, minced, rinsed with cold 0.15 M NaCl and homogenized in Tris buffer. Lung lavage was centrifuged at 4 C for 10 min at 1000 x g to remove cells and debris. The supernatant fluid was freeze-dried and the residue was dissolved in 5 ml of distilled water.

Lipid Analyses

The lipids were extracted from lung tissue and from the freeze-dried, reconstituted lavage fluid using Bligh and Dyer's procedure (6). Aliquots of the total lipid extracts were used to separate neutral lipids, glycolipids and phospholipids by silicic acid column chromatography (7). Only the phospholipid fraction was processed further. Duplicate samples of phospholipid fractions eluted from the silicic acid column were subjected to thin layer chromatographic (TLC) separation on 0.25 mm Silica Gel H plates prepared with 1 mM sodium carbonate. The plates were developed in chloroform/methanol/water/acetic acid (25:15:4:1.75, v/v) containing 0.02% BHT as an antioxidant. After a brief (15-20 sec) exposure to iodine vapors, the spots for phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol + phosphatidylserine (PI + PS) and sphingomyelins (SM) were scraped off and transferred to glass tubes with teflon-lined screw caps. Phospholipids were extracted twice with 3 ml of methanol/chloroform/water (2:1:0.8, v/v). The 2 extracts were combined and 1.6 ml each of chloroform and water was added to separate the chloroform phase from the aqueous phase. The chloroform phase, containing the phospholipids, was used for the determination of phosphorus content using Bartlett's method (8). The recovery of phospholipid standards from the TLC plates using this extraction procedure was 90-95%.

To determine the fatty acid composition of PC, PE and PI + PS fractions, separate TLC plates were developed as described above, except under nitrogen to prevent the oxidation of polyunsaturated fatty acids. Phospholipid spots were extracted as above and transesterified with 14% boron trifluoride-methanol according to the method developed by Morrison and Smith (9). Using a flame ionization detector, the fatty acid composition of total lipids, phospholipids and individual phospholipid fractions was determined by gas chromatography (GC) on 10% SP-2330 glass column (180 x 0.2 cm). Isothermal and temperature programming conditions were used to separate fatty acid methyl esters (FAME). Pure standards of FAME, log retention time vs carbon number and microhydrogenation techniques were used for the identification of fatty acids. Peak areas were calculated using a Hewlett Packard Electronic Integrator (HP #3380 A).

Using the method of Mason et al. (10), the contents of the surfactant DPPC in lung tissue were determined in total lipid extracts after oxidation with osmium tetraoxide and column chromatography. An aliquot of the column eluate was used for phosphorus assay. Another aliquot was transesterified and the fatty acid composition was determined by GC as described above. The values obtained for DPPC from the column eluate by phosphorus assay were corrected according to the proportions of palmitic acid as determined by GC. The protein content of lung homogenates was determined by the biuret reaction (11).

Statistical Analyses

Values in each of the 3 experimental groups (HCO, butter and safflower oil) were compared with the corresponding values in the control group (corn oil) using Student's t test.

RESULTS

The final body weights of rats fed an EFA-deficient diet (7% HCO) were significantly lower ($P<0.001$) compared with their controls (319.8 ± 11.1 g vs 514.0 ± 26.9 g, mean ± SE). No significant difference was found in body weights of rats fed 10% butter or 10% safflower oil compared with the control (586.0 ± 23.7 g for butter and 571.3 ± 19.4 g for safflower oil). Similarly, lung weight was lower in rats fed the EFA-deficient diet compared with their controls (1.33 ± 0.13 g vs 2.36 ± 0.13 g). No difference was found in lung weights of the other 2 groups.

The fatty acid composition of lung tissue phospholipids is shown in Table 1. In rats fed the diet containing HCO, the proportions of 18:1, 20:3ω9 and 22:3ω9 were significantly higher whereas those of 18:2, 20:4ω6 and 22:4ω6 were lower compared with the propor-

TABLE 1

Fatty Acid Composition of Phospholipids of Lungs from Rats Fed Diets Containing Different Lipids

Fatty acid	Dietary lipids			
	7% CO	7% HCO	10% Butter	10% Safflower oil
16:0 DMA	4.0 ± 0.94	5.3 ± 0.64	6.5 ± 0.38[a]	3.0 ± 1.07
16:0	10.7 ± 2.0	13.1 ± 2.23	17.3 ± 0.77[a]	24.3 ± 1.69[b]
16:1	–	4.5 ± 2.16	0.4 ± 0.33	–
18:0 DMA	0.9 ± 0.30	–	2.2 ± 0.08[b]	1.3 ± 0.81
18:0	19.3 ± 0.74	17.5 ± 0.13	23.5 ± 1.21[a]	24.8 ± 0.16[c]
18:1	14.6 ± 0.27	22.0 ± 0.32[c]	25.9 ± 0.74[c]	23.3 ± 1.25[c]
18:2	7.8 ± 0.46	0.4 ± 0.03[c]	0.6 ± 0.08[c]	8.0 ± 0.19
20:3ω9	–	22.1 ± 0.78[c]	2.5 ± 0.48[c]	–
20:4ω6	26.9 ± 1.36	4.2 ± 0.14[c]	19.4 ± 1.0[b]	15.9 ± 0.97[c]
22:3ω9	0.7 ± 0.06	8.9 ± 0.25[c]	–[c]	–[c]
22:4ω6	10.4 ± 0.87	0.9 ± 0.06[c]	–[c]	–[c]

Values are area percent (mean ± SEM of 4 rats per group).
DMA = Dimethyl acetal.
– indicates trace.
[a]$P < 0.05$.
[b]$P < 0.01$.
[c]$P < 0.001$.

TABLE 2

Fatty Acid Composition of Total Lipids of Lung Lavage from Rats Fed Different Dietary Lipids

Fatty acid	Dietary lipids			
	7% CO	7% HCO	10% Butter	10% Safflower oil
14:0	2.5 ± 0.35	2.9 ± 0.23	2.8 ± 0.28	1.5 ± 0.06[a]
16:0	52.7 ± 4.71	56.5 ± 1.34	62.2 ± 3.61	68.1 ± 1.79[a]
16:1	9.2 ± 0.15	17.8 ± 0.38[c]	15.3 ± 0.52[c]	7.2*
18:0	6.3 ± 1.64	2.9 ± 0.4	3.1 ± 0.39	3.9 ± 0.13
18:1	10.8 ± 1.88	13.1 ± 0.7	13.0 ± 1.13	5.5 ± 0.79[a]
18:2	10.0 ± 1.09	0.2 ± 0.09[c]	1.2 ± 0.10[c]	11.3 ± 0.91
20:3ω9	0.3 ± 0.10	5.1 ± 0.25[c]	1.4 ± 0.17[b]	0.3 ± 0.05
20:4ω6	7.6 ± 2.15	0.6 ± 0.08[aa]	3.6 ± 0.18	5.8 ± 0.48

Values are area percent (mean ± SEM of 4 rats per group).
*average of 2 values.
[a]$P < 0.05$.
[aa]$P < 0.02$.
[b]$P < 0.01$.
[c]$P < 0.001$.

tions of these fatty acids in the control group fed corn oil. The differences in the fatty acid composition of phospholipids in the lungs between the control group and the one fed 10% butter were similar to those observed between the control and the group fed HCO. The levels of 16:0, 18:0 and their dimethylacetals (16:0 DMA, 18:0 DMA) were higher in the group fed butter compared with the control group fed corn oil. In the lung phospholipids of rats fed 10% safflower oil, the proportions of 16:0, 18:0 and 18:1 were higher whereas those of 20:4ω6 and 22:4ω6 were lower than the proportions of these fatty acids in the control group. The data on fatty acid composition of lung tissue total lipids (not shown) also reflected differences similar to those observed in phospholipids between the control group and the 3 experimental groups.

The results of the fatty acid composition of total lipids of lung lavage as affected by different diets are shown in Table 2. Palmitic acid constituted 52-68% of the total fatty acids. The proportions of 16:1 and 20:3ω9 were significantly higher and those of 18:2 and 20:4ω6 were significantly lower in the group fed HCO compared with those fed corn oil. In the total lipids of lung lavage obtained from rats fed butter, the levels of 16:1 and 20:3ω9 were higher and those of 18:2 were lower compared

TABLE 3

Fatty Acid Composition of Lung Lavage Phospholipids from Rats Fed Different Dietary Lipids

Fatty acid	Dietary lipids			
	7% CO	7% HCO	10% Butter	10% Safflower oil
16:0	47.3 ± 6.62	50.6 ± 1.38	48.6 ± 1.62	43.9 ± 0.88
16:1	–	–	7.7 ± 1.07[c]	–
18:0	10.6 ± 1.23	6.2 ± 0.75[a]	10.1 ± 0.52	11.5 ± 0.35
18:1	17.3 ± 2.63	25.4 ± 0.98[a]	25.6 ± 1.93[a]	14.7 ± 0.96
18:2	9.6 ± 1.80	0.6 ± 0.11[b]	0.6 ± 0.16[b]	12.2 ± 0.91
20:3ω9	0.1 ± 0.1	8.9 ± 0.48[c]	0.9 ± 0.15[b]	–
20:4ω6	7.4 ± 1.43	0.9 ± 0.18[b]	4.3 ± 0.68	6.4 ± 0.31

Values are area percent (mean ± SEM of 4 rats per group).
–indicates trace.
[a]$P < 0.05$.
[b]$P < 0.01$.
[c]$P < 0.001$.

TABLE 4

Phospholipid Composition of Lung in Rats Fed Different Dietary Lipids

Dietary lipid	PC	PE	PI + PS	SM	DPPC
	(% phospholipid phosphorus)				
7% CO	55.8 ± 3.98	11.8 ± 1.85	7.4 ± 0.41	9.5 ± 0.57	30.8 ± 1.49
7% HCO	45.9 ± 1.41	14.4 ± 1.67	7.5 ± 0.35	10.4 ± 1.11	15.6 ± 0.63[c]
10% Butter	49.3 ± 2.69	12.1 ± 1.46	6.2 ± 0.53	7.8 ± 0.21[a]	22.0 ± 1.15[b]
10% Safflower oil	53.7 ± 0.97	11.2 ± 1.57	7.6 ± 1.89	11.6 ± 1.06	29.6 ± 1.51

Values are means ± SEM of 4 rats per group.
[a]$P < 0.05$.
[b]$P < 0.01$.
[c]$P < 0.001$.

with the corresponding fatty acid levels in the group fed corn oil. The fatty acid patterns of total lipids of lung lavage were similar in groups fed safflower oil vs corn oil except for lower levels of 14:0 and 18:1 and higher levels of 16:0 in the former group.

The results of the fatty acid composition of lavage phospholipids are presented in Table 3. The levels of 18:0, 18:2 and 20:4ω6 were lower, whereas those of 18:1 and 20:3ω9 were higher in lavage of rats fed the EFA-deficient diet compared with the controls. When comparing the group fed the diet containing butter with the one fed corn oil, the proportions of 16:1, 18:1, and 20:3ω9 were relatively higher in the former group. the levels of 18:2, on the other hand, were significantly lower in the group fed butter. The phospholipids of lung lavage from the group fed butter contained relatively high levels of 16:1. The other groups had only trace amounts of this fatty acid. There was no significant difference in the fatty acid composition of lavage phospholipids in rats fed safflower oil vs corn oil.

Phospholipid composition of lung tissue is shown in Table 4. PC constituted 46-56% of the total phospholipids. DPPC was 15-31% of the total lipid phosphorus, accounting for a major portion of PC in the lung. DPPC levels of rats fed HCO were reduced 50% compared with rats fed the control diet. Similarly, DPPC levels in lung phospholipids of rats fed butter were significantly lower ($P < 0.01$) when compared with DPPC levels in the group fed corn oil. PE, PI + PS, and SM constituted the other major phospholipids in the lung. The levels of SM were slightly lower in the group fed butter compared with those fed corn oil. Except for DPPC and SM, no significant differences were found in the levels of various phospholipids in any of the dietary groups.

The fatty acid composition of lung tissue PC fractions is shown in Table 5. Palmitic acid accounted for 54-77% of the total fatty acids in this phospholipid fraction. The proportions of 16:0, 18:2 and 20:4ω6 were lower, whereas those of 16:1, 18:1 and 20:3ω9 were higher in the group fed HCO compared with those fed corn oil. In the lung PC fraction of rats fed

TABLE 5

Fatty Acid Composition of Lung Phosphatidylcholine in Rats Fed Different Lipids

Fatty acid	Dietary lipids			
	7% CO	7% HCO	10% Butter	10% Safflower oil
14:0	6.8 ± 0.66	2.6*	2.3 ± 0.22[c]	1.1 ± 0.23[c]
16:0	70.1 ± 1.37	54.4 ± 1.55[c]	63.4 ± 4.30	77.1 ± 0.67[b]
16:1	0.6 ± 0.33	13.9 ± 2.70[b]	6.9 ± 3.97	–
18:0	6.0 ± 0.45	5.4 ± 1.24	6.0 ± 0.37	5.9 ± 0.43
18:1	9.2 ± 0.23	19.3 ± 2.10[b]	14.5 ± 0.78[c]	6.8 ± 0.21[c]
18:2	4.0 ± 0.59	–[c]	1.5 ± 0.19[b]	5.5 ± 0.13
20:3ω9	0.3 ± 0.06	5.0 ± 0.26[c]	0.9 ± 0.15[b]	0.3 ± 0
20:4ω6	2.7 ± 0.64	–[b]	3.8 ± 0.09	2.7 ± 0.11

Values are area percent (mean ± SEM of 4 rats per group).
*Average of two values.
–indicates trace.
[b]$P < 0.01$.
[c]$P < 0.001$.

butter, the levels of 14:0 and 18:2 were lower, whereas those of 18:1 and 20:3ω9 were higher compared with the levels of these fatty acids in the lung PC fraction of rats fed corn oil. The levels of 16:0 were lower whereas those of 16:1 were higher in the group fed butter. However, because of high variability, these differences were not significant. The levels of 16:0 were higher whereas those of 14:0 and 18:1 were lower in the lung PC fraction of rats fed safflower oil compared to the corn oil group.

The fatty acid patterns of PE and PI + PS fractions (data not shown) of lung tissue generally reflected similar differences between the control group and each of the 3 experimental groups as seen for the PC fraction. Increases in the proportions of 18:1, 20:3ω9 and 22:3ω9 and decreases in those of 18:2, 20:4ω6 and 22:4ω6 were generally observed in the group fed the diet containing HCO compared with the controls. The fatty acid composition of PE and PI + PS fractions of lung tissue from rats fed 10% butter was somewhat similar to those fed HCO but the differences between the 2 groups (corn oil vs butter) were generally less pronounced.

DISCUSSION

Two nutritional models, feeding saturated vs unsaturated fats and an EFA deficiency model, were used in a feeding study to determine their effects on the composition of lung lipids. The results show some similarity in terms of the effects on lung lipids between the 2 nutritional models. This similarity was reflected in fatty acid composition and in the lung surfactant, DPPC, the levels of which in the lungs were drastically reduced in EFA deficiency, and by feeding a diet containing 10% butter. Changes characteristic of EFA deficiency such as an increase in the levels of 16:1 and 18:1, a decrease in 18:2 and 20:4ω6 and an accumulation of 20:3ω9 were observed in the fatty acid composition of total lipids and phospholipids of the lung tissue and lavage of rats fed diets containing HCO. Similar changes in fatty acid composition were also observed in the lung tissue and lavage of rats fed diets containing butter. However, the changes in the fatty acid composition of total lipids and phospholipids in rats fed butter were not quite as great as in the EFA-deficient group fed HCO diet. This is mainly because butter which contained about 2% linoleic acid, when fed at a level of 10%, still supplied about 0.4% of the total calories as linoleic acid. Such a diet would therefore be marginally deficient in EFA, since the requirements of EFA for growing rats have been estimated to be 1-2% of the total calories (12).

Although the diet supplied marginal levels of linoleic acid in the group fed 10% butter, the rats fed this diet were not deficient in EFA since their body weight gains were essentially similar to those fed 7% corn oil and much higher than those fed 7% HCO. In addition the ratios of 20:3ω9 to 20:4 in the total lipids of lung and lavage of rats fed 10% butter, although higher than in those of rats fed corn oil (0.27 vs 0.09 in lung and 0.39 vs 0.04 in lavage), were still lower than 0.4. A ratio higher than 0.4 in tissue lipids is considered to be indicative of EFA deficiency (12). In the present study, the 20:3ω9 to 20:4 ratio ranged from 6.2 to 8.5 in the total lipids of lung and lavage in the EFA-deficient rats.

The results of our study with the EFA-deficient model are in accordance with the findings of Kyriakides et al. (2) and Nakamura et al. (13) on the reduction in DPPC levels in lung tissue and on the fatty acid patterns of PC. The magnitude of decrease in DPPC content in the lung in the EFA-deficient rats in our study was, however, much greater than in those of Kyriakides et al. (2). This is most likely related to the duration of the feeding study and to the time of initiation of the EFA-deficient diets. Whereas Kyriakides et al. fed their diets for 12-14 weeks to the weanling rats, in our study we initiated feeding the diets to the dams as soon as the rats were born and continued feeding the weaned rats for another 21 weeks after weaning.

The presence of relatively higher levels of palmitic acid in the total lipids of lung lavage as compared with the levels of this fatty acid in lavage phospholipids was somewhat surprissing in view of the fact that most of the palmitic acid in lung lavage is present in phospholipids as DPPC. One possibility for lower levels of 16:0 in lavage phospholipids may be the slight contamination of lavage fluid with blood components, although no traces of blood were observed in lavage during the collection procedure. Blood phospholipids are known to contain substantially lower levels of palmitic acid than lung lavage phospholipids (2,14).

Due to the small amount of lavage available from individual rats, we were unable to fractionate the phospholipid fraction further into various classes of phospholipids. Therefore, the levels of DPPC or the fatty acid composition of specific phospholipids were not determined in lung lavage. A decrease in the palmitate content of lavage PC of EFA-deficient rats has previously been observed (15).

In the present studies, we have shown that the feeding of a saturated fat such as butter at a level of 10% during lactation and post-weaning resulted in a marked decrease in the DPPC levels of the lung. Similar data were obtained in the EFA-deficient group. In view of the marginal EFA deficiency induced by feeding 10% butter, this nutritional model may have a greater relevance in terms of practical human nutrition since marginal EFA deficiency is more likely to exist in the human population than frank EFA deficiency. Marginal EFA deficiency is also known to exist in patients with cystic fibrosis (16-18).

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REFERENCES

1. Gail, D.B., Massaro, G.D., and Massaro, D. (1977) J. Appl. Physiol. 42, 88-92.
2. Kyriakides, E.C., Beeler, D.A., Edmonds, R.H., and Balint, J.A. (1976) Biochim. Biophys. Acta 431, 399-407.
3. Gross, I., Ilic, I., Wilson, C.M., and Rooney, S.A. (1976) Biochim. Biophys. Acta 441, 412-422.
4. Gross, I. (1977) Fed. Proc. 36, 2665-2669.
5. American Institute of Nutrition (1977) J. Nutr. 107, 1340-1348.
6. Bligh, E.G., and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911-917.
7. Rouser, G., Kritchevsky, G., and Yamamoto, A. (1967), in Lipid Chromatographic Analysis, (Marinetti, G.V., ed), Vol. 1, pp. 118-120, Dekker, N.Y.
8. Bartlett, G.R. (1959) J. Biol. Chem. 234, 466-468.
9. Morrison, W.R., and Smith, L.M. (1964) J. Lipid Res. 5, 600-608.
10. Mason, R.J., Nellenbogen, J., and Clements, J.A. (1976) J. Lipid Res. 17, 281-284.
11. Gornall, A.G., Bardawill, C.J., and David, M.M (1949) J. Biol. Chem. 177, 751-766.
12. Holman, R.T. (1960) J. Nutr. 70, 405-410.
13. Nakamura, M., Kawamoto, T., and Akino, T. (1980) Biochim. Biophys. Acta 620, 24-36.
14. Walker, B.L. (1967) J. Nutr. 92, 23-29.
15. Burnell, J.M., Kyriakides, E.C., Edmonds, R.H., and Balint, J.A. (1978) Respir. Phys. 32, 195-206.
16. Bennett, M.J., and Medwakowski, B.F. (1967) Am. J. Clin. Nutr. 20, 415-421.
17. Kuo, P.T., Huang, N.N., and Bassett, D.R. (1962) J. Pediatr. 60, 394-403.
18. Rivers, J.P.W., and Hassam, A.G. (1975) Lancet, 642-643.

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Complete Assignment of the Carbon-13 NMR Spectrum of Oiticica Oil: Interpretation of Chemical Shifts in Conjugated Systems

L. BERGTER and **P.R. SEIDL***, *Instituto Militar de Engenharia, 22.290 Rio de Janeiro, Brazil*

ABSTRACT

All absorptions on the carbon-13 spectrum of α- and β-eleostearic and licanic acid esters are assigned. A combination of off-resonance decoupling and lanthanide-induced shifts coupled to polarization by a remote keto group was necessary to establish the correct order of conjugated olefinic carbon atoms. By themselves, none of these techniques would have sufficed to reach the correct conclusions. Steric effects are accounted for but may lead to wrong results if proper consideration is not given to other factors.

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INTRODUCTION

Saturated and olefinic, unbranched, C_{12}-C_{18}, fatty acids and their glyceride (or other esters) have been the subject of several carbon-13 nuclear magnetic resonance (^{13}C NMR) analyses (1). Chemical shifts for most constituents in oilseeds, their extracts and methyl esters can be assigned by recourse to the proper additive parameters (2). However, the presence of conjugation between double bonds requires a more careful approach, as exemplified in the case of *Fevillea trilobata* (3). The ^{13}C NMR analysis of oils containing conjugated double bonds has recently been reported (4,5), but apart from the two double-bond case (6) and the work mentioned above, there is not much information on the subject in the literature. A sound basis for making assignments seems thus to be lacking.

We have studied the oil from oiticica, *Licania rigida* Benth., Chrysobalanaceae family. This oil, obtained on an industrial scale in Northeastern Brazil, contains licanic (4-oxo-*cis,trans, trans*-9,11,13 octadecatrienoic) and α-eleostearic (*cis,trans,trans*-9,11,13-octadecatrienoic) as well as palmitic, stearic, oleic and linoleic acids (7). The ^{13}C NMR spectrum of licanic acid had not been interpreted before and the keto group in the 4-position allows comparison with spectra from α-eleostearic, β-licanic (4-oxo-*trans,trans, trans*) and β-eleostearic (*trans,trans,trans*-octadecatrienoic) acids. Complete assignment of olefinic carbons was thus possible, indicating that considerations based on steric effects must be treated with proper care.

*To whom correspondence should be addressed.

EXPERIMENTAL PROCEDURES

Oiticica (*Licania rigida* Benth.) seeds were obtained from the Brazil Oiticica Company of Ceará, Brazil. Kernels were removed from the shell manually and oil was extracted from the crushed kernels with petroleum ether (bp 30-50 C) in a Soxhlet. Yields of oil were 39% of the seed and 56% of the kernel.

Proton NMR spectra were run in $CDCl_3$ solution on a Varian XL-100-12 spectrometer operating on continuous wave. ^{13}C NMR spectra were run on a Varian CFT-20 spectrometer with 8k memory operating in the Fourier transform mode. Spectra for 4000 and 5000 Hz were obtained from 4096 points (accuracy of ±0.12 and ±0.10 ppm, respectively). Regions of 0-50 ppm and 125-150 ppm were registered with sweep widths of 1000 and 500 Hz (accuracy of ±0.025 and ±0.0125 ppm), respectively.

Shift reagent, $Pr(fod)_3$, was added to a solution of methyl esters of licanic and β-licanic acids in $CDCl_3$.

^1H-NMR

Licanic acid methyl ester ($CHCl_3$-d_1) δ,0.90 ppm (t, 3H), 1.20-1.80 (m,8H), 2.10 (m,4H), 2.45 (m,2H), 2.60 (m,4H), 3.60 (s,3H), 5.37 (m,1H), 5.60 (m,1H), 5.90-6.50 (m,4H). β-Licanic acid methyl ester ($CHCl_3$-d_1) 0.90 ppm (t,3H), 1.10-1.80 (m,8H), 2.06 (m,4H), 2.42 (m,2H), 2.60 (m,4H), 3.60 (s,3H), 5.20-5.76 (2H), 5.76-6.40 (m,4H).

Oils were transesterified by stirring with 0.02 M methanolic sodium methoxide at room temperature for 3 hr. Methyl esters were separated by column chromatography using silica gel (70-230 mesh, ASTM) with a petro-

TABLE 1

^{13}C Chemical Shifts of Conjugated Octadecatrienoic Acids as Methyl Esters

	c,t,t-9,11,13	*t,t,t*-9,11,13	4-oxo-*c,t,t*-9,11,13	4-oxo-*t,t,t*-9,11,13
C_1 [a]	174.11	174.11	173.88	173.09
C_2 [b]	34.13	34.15	27.78	27.76
C_3 [b]	25.06	25.05	37.07	37.05
C_4	29.21[b]	29.22[b]	208.48[a]	208.48[a]
C_5 [b]	29.21	29.22	42.60	42.53
C_6 [b]	29.21	29.09	23.47	23.37
C_7 [b]	29.79	29.44	29.30	28.96
C_8 [b]	27.87	32.85	27.64	32.55
C_9 [c]	131.66	134.12	130.94	133.41
C_{10} [c]	129.00	130.81	129.25	130.99
C_{11} [c]	126.09	130.93	125.85	130.68
C_{12} [c]	132.97	131.06	133.18	131.19
C_{13} [c]	130.84	130.70	130.69	130.53
C_{14} [b]	134.98	134.30	135.23	134.48
C_{15} [b]	32.58	32.58	32.57	32.55
C_{16} [b]	31.61	31.67	31.57	31.61
C_{17} [b]	22.32	22.32	22.29	22.28
C_{18} [b]	13.94	13.95	13.94	13.94
OCH_3	51.26	51.26	51.67	51.63

[a]Measured at 5000 Hz.
[b]Measured at 1000 Hz.
[c]Measured at 500 Hz.

leum ether/ethyl ether gradient, and analyzed by gas liquid chromatrgrphy (GLC).

GLC

Chromatograms were run on 1m x 3mm columns packed with 1.5% Dexsil 300 on Chromosorb W (80-100 mesh), washed with acid and silanized at 120-270 C, 4 C/min at 30 ml/min nitrogen. Oil composition: 16:0–6.8%; 18:0, 18:1, 18:2–22.6%; 18:3 (conj)–15.1%; 18:3 (oxo, conj)–55.4%.

Conversion to the *trans,trans,trans*-isomers was effected by treatment with iodine in petroleum ether (8).

RESULTS AND DISCUSSION

As inferred from the ^{13}C NMR spectra, no other conjugated unsaturated acids besides α-eleostearic and licanic are present in oiticica oil. Except for those expected in carbonyl and glycerol regions, there are no marked differences between the spectra of triglycerides and monoesters (vide infra).

Assignment of all peaks was carried out on oils, their transesterification products and enriched fractions of methyl esters. Comparison with similar compounds, selective partial carbon-hydrogen decoupling experiments, the use of lanthanide shift reagents and combination thereof afforded complete assignment of absorption using the approaches described below.

Saturated carbon atoms of α- and β-licanic acid methyl esters were assigned by comparison with those of the α- and β-eleostearate ester (Table 1). Increments that could be attributed to the keto group in the 4-position were estimated from 4-oxo-methyl stearate (9), the maximum observed differences were, respectively, +0.22 ppm for C-4 and ±0.14 ppm for the remaining carbon in the case of licanic acid and +0.34 ppm for C-4, +0.21 ppm for C-5 and an upper limit of 0.12 ppm for the remaining carbons of β-licanic acid.

Selective decoupling experiments were run on a pure sample of the methyl ester of licanic acid and an enriched fraction of that of α-eleostearic acid. Double resonance experiments on the proton spectrum of licanic acid methyl ester allow the assignment of H_9, H_{10} and H_{14} to the peaks at 5.32, 5.9 and 5.65, respectively. H_9 suffers the largest influence of the addition of $Pr(fod)_3$, whereas H_{14} is not affected. The remaining olefin peaks lie between 6.0 and 6.5 ppm, as expected from the literature (10).

Selective carbon-hydrogen decoupling is based on irradiation at 3.75, 5.00, 6.25 and 7.50 ppm of the proton spectrum, using variable decoupling power without noise modulation. Irradiation at higher field affects carbon signals at 130.94 and 135.23 ppm more strongly, and that at low field affects those at 125.85 and 133.18 ppm. The former must then correspond to C_9 and C_{14}.

Addition of the lanthanide reagent induces

TABLE 2

Comparison of ^{13}C Chemical Shifts of Double-Bond Carbons of Octadecatrienoic Acids[a]

Carbon number	(*c,t,t*-9,11,13)	(4-oxo-*c,t,t*-9,11,13)	$\Delta\delta c$[(4-oxo-*c,t,t*-9,11,13)-(*c,t,t*-9,11,13)][b]	(*t,t,t*-9,11,13)	(4-oxo-*t,t,t*-9,11,13)	$\Delta\delta c$[(4-oxo-*t,t,t*-9,11,13)-(*t,t,t*-9,11,13)][c]
9	131.64	130.93	− 0.71	134.03	133.33	− 0.70
10	128.88	129.22	+ 0.34	130.65	130.97	+ 0.32
11	126.02	125.85	− 0.17	130.79	130.65	− 0.14
12	132.92	133.16	+ 0.24	130.91	131.14	+ 0.23
13	130.70	130.70	0.00	130.57	130.53	− 0.04
14	134.99	135.17	+ 0.18	134.18	134.34	+ 0.16

[a]Values in ppm.
[b]Measured on the oil.
[c]Measured on a mixture of methyl esters.

the largest shift on the absorption at 130.94 and smaller and almost equal shifts on the absorption at 129.25 and 125.85 ppm. The other signals practically do not move, confirming the assignments of C_9 and C_{14} and suggesting that absorptions at 129.25 and 125.85 should be ascribed to C_{10} and C_{11}. Choice between the two is possible by selective decoupling, since only the absorption at 125.85 ppm is affected by irradiation of the low field region of the proton spectrum (i.e., vicinity of H_{11}).

Selective carbon-hydrogen decoupling experiments run on enriched fractions of the methyl ester of α-eleostearic acid reveal that absorptions follow the same order as those of licanic acid (no inversions are observed). Thus, absorptions corresponding to carbons 9, 10, 11 and 14 may also be assigned. Introduction of a keto group in the 4-position does have a noticeable effect, however, and can be useful in spectral identification, as will be shown subsequently.

Comparing chemical shifts of triglycerides of α-eleostearic acid and licanic acid, it seems that the conjugated system is polarized by the carbonyl group (see Table 2). Thus, C_{10} and C_{14} are shifted to lower fields and C_9 and C_{11} are shifted to higher fields in the presence of a keto group at C_4. This being the case, C_{12} should reveal a downfield increment between 0.34 and 0.18 ppm, and the upfield increment in C_{13} should lie below 0.17 ppm (this value tapers off very rapidly), indicating the corresponding absorptions for C_{12} and C_{13}.

Assignment of the *trans,trans,trans*-isomers (β-licanic and β-eleostearic acids) follows the same type of approach. Here the hydrogen spectrum reveals 2 protons at 5.3-5.8 ppm and 4 protons at 5.8-6.2 ppm, the former corresponding to H_9 and H_{14}, respectively (10). Selective decoupling on β-eleostearic acid reveals that irradiation of the high field end of the proton spectrum does affect the carbon absorptions at 134.12 and 134.30 ppm, thus they must correspond to C_9 and C_{14} (in this order, as is revealed for β-licanic, below). Irradiation at low field shows a different effect on the remaining protons. The absorptions at 131.06 and 130.93 ppm are more strongly enhanced than those at 130.81 and 130.70 ppm, revealing that each of the 2 pairs of carbon atoms must correspond to a different type of proton.

On β-licanic acid, selective decoupling at high field affects absorptions at 134.48 and 133.41, the later more strongly (this relative effect is inverted on irradiation at low field). The absorption at 133.41 should correspond to C_9 since it also suffers the largest induced shift in presence of $Pr(fod)_3$. On irradiation at low field, the remaining absorptions reveal the same behavior as in the β-eleostearic case, those at 131.19 and 130.68 being more strongly enhanced than those at 130.99 and 130.53. Addition of shift reagent reveals that peaks at 130.68 and 130.99 ppm must correspond to C_{10} and C_{11} but does not indicate which is which.

Assuming that the keto group in 4-position does not invert the relative order of proton absorptions (which is valid for *cis,trans,trans*-isomers in which the respective proton spectra are identical in the olefinic region and H_9, H_{10} and H_{14} display the same chemical shifts for both cases) (10), the same trends relative to the polarization of the double bonds seem to be operative. Thus, C_{10} and C_{12} can be differentiated from C_{11} and C_{13} within their respective groups, since the former would be shifted downfield and the latter upfield in the presence of the 4-keto group (and all the remaining assignments can be made), as shown in Table 2.

The above results imply a correction of assignments made for α- and β-eleostearate esters identified in Fevillea oil (3), revealing that care must be taken in interpretation of steric effects in conjugated olefin systems. Chemical shift differences between α- and β-isomers indicate the type of steric effect that may be expected. The relatively large (ca. 5 ppm) upfield shifts at C_8 and C_{11} are similar to those observed in the case of two conjugated double bonds (11) and are ascribed to the close contact between their respective protons (the *cis-cis* case is still larger, ca. 6 ppm) (12).

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REFERENCES

1. Wenkert, E., Buckwalter, B.L., Bursitt, I.R., Rosler, M.I., Garic, J., Gottlieb, H.E., Hagaman, E.W., Schell, F.M., Workulich, P.M., Zheleva, A., (1976) in Topics in Carbon-13 NMR Spectroscopy (Levy, G.C., ed.) Vol. 2, pp. 81-117, John Wiley & Sons, New York.
2. Leal, K. Z., Costa, V.E.U., Seidl, P.R., Campos, M.P.A., and Colnago, L.A. (1981) Cienc. Cultu. 33, 1475-1484.
3. Tulloch, A.P., and Bergter, L. (1979) Lipids 14, 996-1006.
4. Gowrikumar, G., Mani, V.V.S., Chandrasekhara, R.T., Kaimal, T.N.B., and Laksminarayana, G. (1981) Lipids 16, 558-559.
5. Tulloch, A.P. (1982) Lipids 17, 544-550.
6. Bus, J., Sies, I., and Lie Ken Jei, M.S.F. (1977) Chem. Phys. Lipids 18, 130-144.
7. Kaufmann, H-P., and Sud, R.K. (1960) Fette Seifen Anstrichm. 3, 160-164.
8. Hopkins, C.Y. (1972) in Topics in Lipid Chemistry (Gunstone, F.D., ed.) Vol. 3, pp. 37-87, Logos Press, London.
9. Tulloch, A.P. (1977) Can. J. Chem. 55, 1135-42.
10. Suzuki, O., Tetsutaro, H., Hayamizu, K., and Yamamoto, O. (1970) Lipids 5, 457-462.
11. Bus, J., Sies, I., and Lie Ken Jei, M.S.F. (1976) Chem. Phys. Lipids 17, 501-518.
12. Batchelor, J.G., Cushley, R.J., and Prestegard, J. (1974) J. Org. Chem. 12, 1698-1705.

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The Dietary Regulation of Acyltransferase and Desaturase Activities in Microsomal Membranes of Rat Liver

E.L. PUGH and M. KATES*, *Department of Biochemistry, University of Ottawa, Ottawa K1N 9B4, Canada*

ABSTRACT

Dietary manipulation produces marked alterations in desaturase activities of rat liver microsomes with no concomitant changes in acyltransferase activities. Desaturation of stearoyl-CoA (Δ9-desaturase), linoleoyl-CoA (Δ6-desaturase), eicosatrienoyl-CoA (Δ5-desaturase) and eicosatrienoyl-phosphatidylcholine (Δ5-desaturase) was elevated in animals fed a corn oil diet and lowered in those fed a coconut oil diet compared to control animals. The Δ5-desaturase activities were also lowered in starved animals and elevated in starved animals refed a fat-free diet. However, no changes in acyl-CoA:1-acyl-*sn*-glycero-3-phosphocholine acyltransferase activity were observed in the membranes of animals maintained on any of the dietary regimens studied. These observations suggest that the desaturases of rat liver microsomes are regulated independently of the acyltransferases and that desaturation of eicosatrienoyl-phosphatidylcholine is regulated at the level of the desaturase itself and not by availability of the phospholipid substrate.

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INTRODUCTION

Many studies have been made of the metabolic regulation of the stearoyl-CoA desaturase of liver microsomes. Several factors including allosteric factors (1), soluble activators (2), lipid factors (3,4) and modulation of the level of desaturase protein (5-7) have all been implicated in control of this enzyme system. More recently, evidence has been obtained to suggest that the activity is also closely related to changes in the lipid environment. For example, stearoyl-CoA desaturase activity of chicken liver is increased by injection of the hormone, 17β-estradiol, and this increase is related to a decrease in membrane fluidity as measured by fluorescence polarization of the probe diphenylhexatriene (8). Also, studies of a stearoyl-CoA desaturase reconstituted by highly purified protein components and lipids indicated that desaturase activity was dependent on the nature of the lipid used in the reconstitution experiments (9).

In spite of the extensive studies on regulation of the stearoyl-CoA (Δ9-) desaturase, little is known about regulation of the eicosatrienoyl-CoA (Δ5-) desaturase of rat liver. Previous studies from this laboratory have suggested that both [1-^{14}C]eicosatrienoyl-CoA and 1-acyl-2-[^{14}C]eicosatrienoyl-*sn*-glycero-3-phosphocholine (20:3-PC) may serve as substrates for the liver microsomal Δ5-desaturase (10,11). The levels of these Δ5-desaturase activities, like that of the Δ9-desaturase, were found to vary with changes in the lipid environment (10-12). For example, starving animals and refeeding a fat-free diet resulted in increased activity of the Δ5-desaturase with both the phospholipid and acyl-CoA substrates (10). These increases in activity were accompanied by a decreased level of polyenoic fatty acids in the microsomal membrane of the starved-refed rat and a higher transition temperature of the microsomal phospholipids as measured by fluorescence polarization of *cis*- and *trans*-parinaric acid probes (13,14). These data suggest that the microsomal phospholipids of the starved-refed rat were in a less fluid state than those of control and starved rats and that this decrease in fluidity was correlated with an increase in desaturase activity.

In addition to changes in the lipid environment, other factors such as control of enzyme synthesis and availability of substrate may regulate the Δ5-desaturases of rat liver microsomes. In view of the previous report (15) that the specific activities of acyltransferases increased in starved-refed animals, we have examined the possibility that formation of the 20:3-PC phospholipid substrate via acyltransferase activity may be a factor in the regulation of the phospholipid desaturase.

MATERIALS AND METHODS

[1-^{14}C]Stearic (50 Ci/mol), [1-^{14}C]linoleic (52 Ci/mol) and [1-^{14}C]homo-γ-linolenic (all-*cis*-8,11,14-eicosatrienoic) (58.8 Ci/mol) acids

*To whom correspondence should be addressed.

Abbreviations: 1-acyl-GPC, 1-palmitoyl-*sn*-glycero-3-phosphocholine; DTNB, 5,5'-dithiobis (2-nitrobenzoic acid).

were obtained from New England Nuclear (Boston, MA) and were more than 98% radiochemically pure. 1-Palmitoyl-*sn*-glycero-3-phosphocholine (1-acyl-GPC) was a product of Serdary Research Laboratories (London, Canada) and gave one spot on thin layer chromatography (chloroform/methanol/water, 65:35:5, v/v/v). Snake venom from *Crotalus adamanteus* and 5,5'dithiobis (2-nitrobenzoic acid) (DTNB) were obtained from Sigma Chemical Co. (St. Louis, MO). The fat-free test diet, corn oil and coconut oil were products of Nutritional Biochemicals (Cleveland, OH).

[1-^{14}]Stearoyl-CoA (0.8 Ci/mol), [1-^{14}C]-linoleoyl-CoA (0.8 Ci/mol) and [1-^{14}C]eicosatrienoyl-CoA (2.1 Ci/mol) were prepared from the N-hydroxysuccinimide esters (16) by the method of Al-Arif and Blecher (17). The fatty acyl-CoA esters obtained had absorbance ratios (A_{232}/A_{260}, thiol absorption/adenine absorption) from 0.52 to 0.62 (theoretical value, 0.573) (18). The concentration of acyl-CoA was calculated from the extinction coefficient of the absorbance at 260 nm (16,400 cm^{-1} M^{-1}) (19).

1-Acyl-2-[^{14}C] eicosatrienoyl-*sn*-glycero-3-phosphocholine (12 Ci/mol) was prepared biosynthetically, using rat liver microsomes, as described previously (10).

Animals and Diets

Male Sprague-Dawley rats (125-150 g) were either fed Purina laboratory chow ad libitum ("control"), starved for 48 hr ("starved") or starved and refed a fat-free diet ("starved-refed") as described by Strittmatter et al. (6). Starved-refed rats were fasted 48 hr, fed Purina laboratory chow for 24 hr, fasted a second 48 hr period and refed with Nutritional Biochemicals' fat-free diet for 20 hr.

In the second group of experiments, spontaneously hypertensive rats of the Okamoto-Aoki strain, weighing 250-300 g, were placed on one of the following diets: fat-free diet ("control"), a fat-free diet supplemented with 20% corn oil ("corn oil-fed") or a fat-free diet supplemented with 20% coconut oil ("coconut oil-fed"). The rats were fed ad libitum for a period of 6-16 weeks. The rats that were fed the fat-free diet had no symptoms of essential fatty acid deficiency (fur texture changes), even after 16 weeks of consuming a fat-free diet.

All animals were killed by decapitation and liver microsomes were prepared as described previously (10).

Assay Procedures

Acyltransferase activity was measured at room temperature by the spectrophotometric assay of Lands and Hart (20). In this assay, CoASH liberated by enzyme activity reduces DTNB to form 2-nitro-4-thiobenzoic acid that has an absorption maximum at 414 nm and an extinction coefficient of 13,600 cm^{-1} M^{-1} (21). The reaction mixture in the sample cuvette (1 ml) contained 0.1 M Tris-HCl (pH 7.2), 0.3 mM DTNB, 50 μM 1-acyl-GPC, 20 μM [1-^{14}C]eicosatrienoyl-CoA (2.1 Ci/mol) and microsomal protein as indicated in the tables and figure legends. The mixture in the reference cuvette was identical, except that 1-acyl-GPC was omitted and replaced by an equal volume of distilled water. Incubations were carried out at room temperature for 3 min. The reaction was followed by measuring the increase in absorbance at 414 nm using a Unicam SP1800 recording spectrophotometer.

Fatty acyl-CoA transferase activity was also measured using a radioactive tracer assay at room temperature or at 37 C. The reaction mixture was identical to that of the spectrophotometric assay. At the end of the incubation period, the reaction was terminated by extraction of the lipids with methanol/chloroform (2:1, v/v) according to the method of Bligh and Dyer (22). Total lipids were then separated into neutral lipids and phospholipids on silicic acid columns by elution with chloroform/methanol (9:1, v/v) and methanol, respectively, as described previously (14). Both the neutral lipid and the phospholipid fractions were then examined by thin layer chromatoggraphy (TLC) on Silica Gel H plates in the solvent system chloroform/methanol/water (65:35:5, v/v/v) as described previously (23) using authentic samples of lecithin, lysolecithin and fatty acids as markers. The lipid zones were visualized with iodine vapor, scraped and radioactivity determined in a liquid scintillation counter.

Desaturation of fatty acyl-CoA esters and 1-acyl-2-[^{14}C]eicosatrienoyl-*sn*-glycero-3-phosphocholine was measured in a reaction mixture (1 ml) containing 100 mM potassium phosphate (pH 7.2), 100 mM sucrose, 10 mM NADH, 0.5-2 mg of microsomal protein and either 30 μM acyl-CoA or 30 μM 1-acyl-2-[^{14}C]eicosatrienoyl-*sn*-glycero-3-phosphocholine plus 0.1% Triton X-100. Incubations were at 37 C for 15 min. Reaction products were extracted, methanolyzed, and the fatty acyl methyl esters analyzed by $AgNO_3$-TLC as described previously (10). All desaturase assays were carried out at 2 or more concentrations of protein to ensure that the rate of desaturation was proportional to the amount of microsomal protein used.

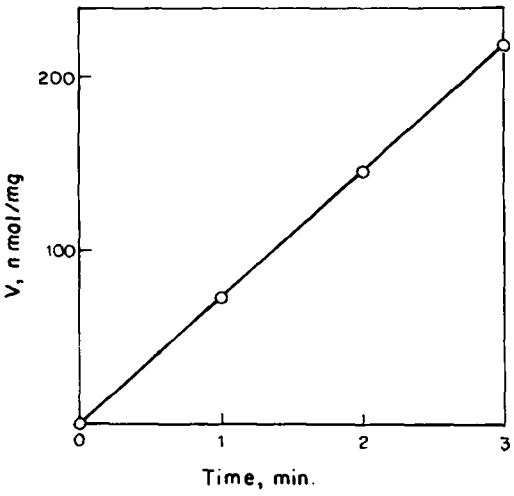

FIG. 1. Time course of acyl-CoA:1-acyl-*sn*-glycero-3-phosphocholine acyltransferase. The spectrophotometric assay for release of CoASH is described under Assay Procedures. The reaction mixture contained 40 μg microsomal protein; incubations were carried out at room temperature for time periods indicated. Data are corrected for acyl-CoA hydrolase activity.

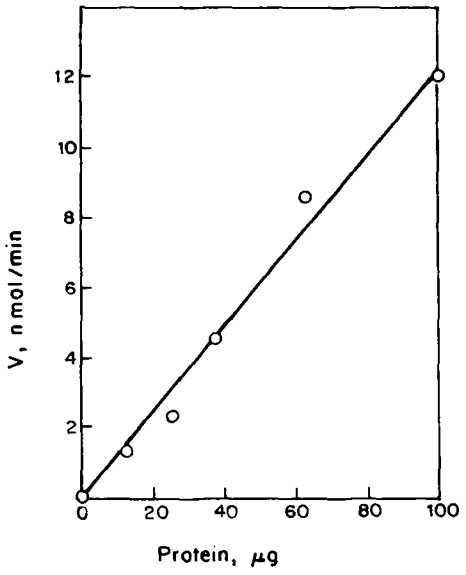

FIG. 2. Acyl-CoA:1-acyl-*sn*-glycero-3-phosphocholine acyltransferase activity as a function of protein concentration. The spectrophotometric assay for release of CoASH was as described under Assay Procedures and incubations were at room temperature for 3 min. Data are corrected for acyl-CoA hydrolase activity.

Protein was determined by the method of Lowry et al. (24), using bovine serum albumin as standard.

Determination of Positional Distribution of Eicosatrienoate Incorporated into Phosphatidylcholine

The location of [^{14}C]eicosatrienoate in phosphatidylcholine was determined by treatment with phospholipase A_2 from *C. adamanteus* venom as described elsewhere (14). The lipid products were extracted by the Bligh-Dyer procedure (22) and again chromatographed on a column of silicic acid as described above. The reaction products, free fatty acids and lysolecithin, were obtained in the chloroform/methanol (9:1) and methanol eluates, respectively. The amount of [^{14}C]eicosatrienoate in each eluate was determined in a liquid scintillation counter.

Lipid Analyses

Total lipids were extracted by the method of Bligh and Dyer (22), transesterified in 2.5% methanolic HCl, and the resulting methyl esters analyzed by gas liquid chromatography on SP-2330 at 190 C. Peaks were identified by comparison with authentic methyl ester standards and peak areas measured by the retention time x peak height method of Carroll (25).

RESULTS

Properties of Acyl-CoA: 1-Acyl-*sn*-glycero-3-phosphocholine Acyltransferase

The acyltransferase had a broad pH optimum in the range 7.0-8.0 and initial velocity was linear with time for 3 min (Fig. 1). Under the conditions of the assay, the activity was also proportional to microsomal protein concentration to ca. 100 μg/ml (Fig. 2).

Substrate Requirements

The effect of various concentrations of 1-acyl-GPC on fatty acyl-CoA transferase activity was examined (Fig. 3). Maximal activity was observed at ca. 50 μM 1-acyl-GPC. The reaction showed typical Michaelis-Menten kinetics with apparent K_m and V_{max} values for 1-acyl-GPC of 67 μM and 233 nmol/min/mg, respectively. The acyltransferase activity was also measured as a function of eicosatrienoyl-CoA concentration (Fig. 4). At saturating concentrations of 1-acyl-GPC, the activity was found to be optimal at 10 μM acyl-CoA. Apparent K_m and V_{max} values for eicosatrienoyl-CoA obtained from Lineweaver-Burk plots were 6 μM and 161 nmol/min/mg, respectively.

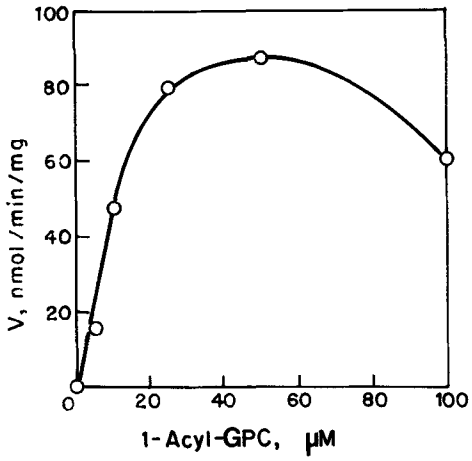

FIG. 3. Effect of 1-acyl-GPC concentration on acyl-CoA:1-acyl-*sn*-glycero-3-phosphocholine acyltransferase activity. The spectrophotometric assay for release of CoASH is described under Assay Procedures. 1-Acyl-GPC concentrations were varied as indicated. The reaction mixture contained 40 μg microsomal protein; incubations were at room temperature for 3 min. Data are corrected for acyl-CoA hydrolase activity.

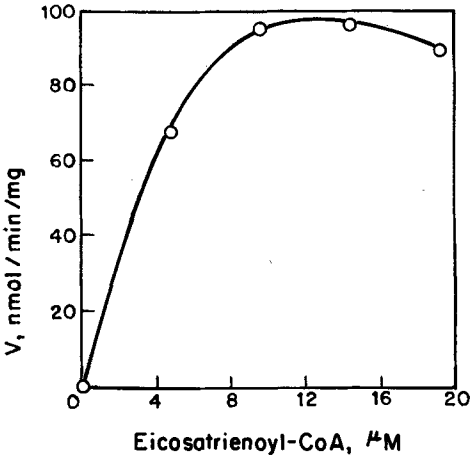

FIG. 4. Effect of eicosatrienoyl-CoA concentration on acyl-CoA:1-acyl-*sn*-glycero-3-phosphocholine acyltransferase activity. The spectrophotometric assay is described under Assay Procedures. Eicosatrienoyl-CoA concentrations were varied as indicated. The reaction mixture contained 60 μg microsomal protein; incubations were at room temperature for 3 min.

Comparison of Spectrophotometric and Radioactive Tracer Assays of Acyltransferase Activity

The acyltransferase activity obtained in the studies reported above by the spectrophotometric assay was compared with the activity obtained with a radioactive tracer assay (Table 1). The activity calculated from the 2 assay methods was found to agree within ±4-8%.

TABLE 1

Comparison of Spectrophotometric and Radioactive Tracer Assays of Acyltransferase Activity

Protein (μg)	Spectrophotometric assay[a]	Radioactive tracer assay[b]
	Acyltransferase activity (nmol/min)	
0	0	0
38	2.7	2.5
50	3.6	3.4
63	5.2	5.0

[a]The reaction mixture in the sample cuvette contained 0.1 M Tris-HCl (pH 7.2), 0.33 mM DTNB, 50 μM 1-acyl-GPC, 20 μM [1-^{14}C]eicosatrienoyl-CoA (2.1 Ci/mol) and microsomal protein as indicated above. The mixture in the reference cuvette was identical except that 1-acyl-GPC was omitted and replaced by an equal volume of distilled water. Reaction was at room temperature for 3 min. The reaction was followed by measuring the increase in absorbance at 414 nm.

[b]The reaction mixture was identical to that in the sample cuvette described above. Reaction was at room temperature for 3 min. The reaction was stopped by adding chloroform/methanol, and radioactive products were isolated by column chromatography and thin layer chromatography as described under Assay Procedure.

The rat liver microsomes were also assayed for fatty acyl-CoA hydrolase which competes with fatty acyl-CoA transacylase for the CoA esters (20). Even though the activity of this enzyme is corrected for by the spectrophotometric assay, it was important to determine whether it was consuming a significant amount of the CoA esters under the experimental conditions used to measure fatty acyl-CoA transferase activity. When fatty acyl-CoA hydrolase activity for the CoA ester of eicosatrienoate was measured by the spectrophotometric method in the absence of 1-acyl-GPC, it was found that this activity was ca. 8% of acyl-CoA: 1-acyl-*sn*-glycero-3-phosphocholine acyltransferase activity.

Products of Acyl-CoA: 1-Acyl-*sn*-glycero-3-phosphocholine Acyl Transferase

The products of the fatty acyl-CoA transferase were determined by the radioactive tracer assay using [1-^{14}C]eicosatrienoyl-CoA and 1-acyl-GPC; 91 and 8% of the radioactivity was found in phosphatidylcholine and free fatty acids, respectively (Table 2). Only trace amounts of other phospholipids including phosphatidylethanolamine and phosphatidylserine/phosphatidylinositol were found, indicating

TABLE 2

Incorporation of Radioactivity from [1-^{14}C]Eicosatrienoyl-CoA into Phospholipids[a]

Phospholipid zone	Radioactivity (dpm)	% of total
Free fatty acid	1,371	8.4
Phosphatidylethanolamine	30	0.2
Phosphatidylserine/phosphatidylinositol	35	0.2
Phosphatidylcholine	14,896	91.0
Sphingomyelin	26	0.2
Lysophosphatidylcholine	20	0.1

[a]The reaction mixture, containing 50 μg microsomal protein, was as described in Table 1. Incubation was carried out at room temperature for 3 min. Results are the mean of duplicate determinations with deviation of ±2% and have been corrected for background.

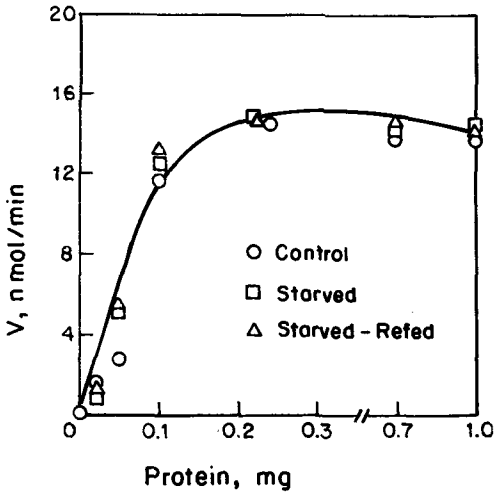

FIG. 5. Effect of nutritional state on acyl-CoA:1-acyl-*sn*-glycero-3-phosphocholine acyltransferase activity. Acyltransferase activities were measured in microsomal membranes isolated from control (○), starved (□), and starved-refed (△) rats by the radioactive tracer assay described under Assay Procedures. Protein concentrations were varied as indicated; incubations were at room temperature for 3 min. Values represent the average of 2 independent determinations with deviation ±2%.

that under these experimental conditions [^{14}C]eicosatrienoate is preferentially incorporated into phosphatidylcholine compared to other phospholipids.

Distribution of Eicosatrienoate Incorporated into Phosphatidylcholine

The phospholipid product recovered from experiments using [1-^{14}C]eicosatrienoyl-CoA as substrate was treated with snake venom phospholipase A_2 to determine the position of incorporation of radioactivity into phosphatidylcholine. The radioactivity was found to be incorporated predominantly at the C-2 position (98%) of the phospholipid.

Effect of Nutritional State on Acyltransferase and Desaturase Activities

Microsomal membranes from starved-refed, starved and control rats prepared as described under Assay Procedures were assayed for acyltransferase activity. The rate of acyltransferase activity was found to be essentially identical in the membranes from rats maintained on these 3 dietary regimens (Fig. 5), when assayed either at room temperature or at 37 C.

The microsomal membranes from control, starved and starved-refed rats were also assayed for desaturation of [1-^{14}C]eicosatrienoyl-CoA and 1-acyl-2-[^{14}C]eicosatrienoyl-*sn*-glycero-3-phosphocholine. As reported previously (10-13), desaturation of both substrates was dependent on the nutritional state of the animal. In control rats, desaturation of eicosatrienoyl-CoA and eicosatrienoyl-glycero-phosphorylcholine was observed at rates of 50 and 20 pmol/min/mg, respectively. In starved-refed rats, desaturation of both acyl CoA and phospholipid substrates was elevated; rates for the eicosatrienoyl-CoA and eicosatrienoyl-phosphatidylcholine desaturase were 130 and 50 pmol/min/mg, respectively, in these microsomal membranes. In contrast, as reported earlier, membranes from starved rats had no detectable desaturase activity with either substrate.

Effect of Diet on Acyltransferase and Desaturase Activities

Acyltransferase activity was also measured in rats fed a fat-free diet or a diet supplemented with either 20% corn oil or 20% coconut oil for 6-9 weeks. The acyltransferase activity was essentially identical in the liver microsomes of these animals irrespective of diet (Fig. 6). Also, it was found that the initial velocity (but not

the extent of the reaction) was essentially the same in these animals as in those that were either starved for a 48 hr period ("starved") or starved and then refed a fat-free diet overnight ("starved-refed") (Figs. 5 and 6).

The microsomal membranes of rats fed the corn oil and coconut oil diets were also assayed for stearoyl-CoA (Δ9-), linoleoyl-CoA (Δ6-) and eicosatrienoyl-CoA (Δ5-) desaturase activities (Table 3). The Δ9-, Δ6- and Δ5-desaturase activities were found to be elevated in animals fed a corn oil diet and lowered in those fed a coconut oil diet compared to the controls.

Fatty Acid Composition of Total Lipids from Microsomal Membranes of Corn Oil- and Coconut Oil-Fed Rats

Fatty acid analyses of total lipids isolated from membranes of rats fed on different diets revealed differences in the proportions of unsaturated fatty acids in the membrane phospholipids (Table 4). The total lipids from microsomal membranes from both corn oil- and coconut oil-fed rats had higher levels of arachidonic acid (20:4) and lower levels of docosahexaenoic (22:6) than the controls after 6-9 weeks. No significant change in 18:2 relative to the controls was observed in microsomes of corn oil-fed rats, but coconut oil-fed rats had significantly lower 18:2 levels. The fatty acid compositions observed resulted in lower double bond indices for these 2 membranes (4.3-4.4) after 6-9 weeks compared to those observed for the control membranes (5.0) (Table 4). It should be noted that after 16 weeks on the corn oil diet both the 20:4 and 22:6 levels increased, resulting in a much higher double bond index (6.9) than at 9 weeks. The levels of 16:0 and 18:0 were not significantly affected by either diet up to 6-9 weeks, but a pronounced decrease in 16:0 level occurred after 16 weeks on the corn oil diet (Table 4).

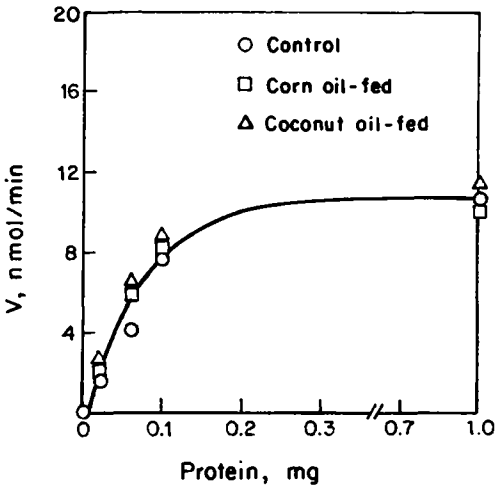

FIG. 6. Effect of diet on acyl-CoA:1-acyl-*sn*-glycero-3-phosphocholine acyltransferase activity. Acyltransferase activities were measured in microsomal membranes isolated from rats fed a fat-free diet (control; ○), a diet supplemented with corn oil (□) or a diet supplemented with coconut oil (△). The radioactive tracer assay is described under Assay Procedures. Protein concentrations were varied as indicated; incubations were at room temperature for 3 min. Values represent the average of 2 independent determinations with deviation ±2%.

DISCUSSION

The present study demonstrates that dietary

TABLE 3

Influence of Diet on Stearoyl-, Linoleoyl- and Eicosatrienoyl-CoA Desaturase Activities of Rat Liver Microsomes

Diet	Rate of desaturation[a]		
	Stearoyl-CoA	Linoleoyl-CoA	Eicosatrienoyl-CoA
		(pmol/min/mg protein)	
Control diet			
6 wk	339 ± 35	85 ± 15	205 ± 23
9 wk	330 ± 34	90 ± 16	215 ± 25
Corn oil diet			
6 wk	426 ± 45	116 ± 15	558 ± 75
9 wk	400 ± 43	120 ± 18	560 ± 70
16 wk	266 ± 26	161 ± 15	554 ± 61
Coconut oil diet			
6 wk	312 ± 33	58 ± 11	151 ± 59
9 wk	300 ± 36	60 ± 15	165 ± 18

[a]Results are given for 3 determinations, using microsomal membranes from pooled livers of 5 rats; incubations were for 15 min at 37 C.

TABLE 4

Fatty Acid Composition of Total Lipids from Liver Microsomal Membranes of Rats Fed Various Diets[a]

Fatty acid	mol %						
	Control diet		Corn oil diet			Coconut oil diet	
	6 wk	9 wk	6 wk	9 wk	16 wk	6 wk	9 wk
14:0	0.2	0.1	0.2	0.1	0.2	1.0	0.6
16:0	20.6	23.9	18.0	19.6	13.3	18.7	20.2
16:1	0.6	nd[b]	0.5	0.1	0.2	0.8	0.1
18:0	19.4	18.3	23.4	21.9	20.4	21.9	22.4
18:1	5.7	7.2	5.9	6.1	6.6	6.9	7.0
18:2	14.8	13.0	14.5	12.6	12.6	11.0	9.2
18:3	0.2	0.1	0.8	0.2	nd	0.3	0.1
20:3	2.9	0.7	1.7	0.1	0.6	2.6	0.6
20:4	26.9	24.9	33.4	32.0	36.9	33.1	30.9
22:5	nd	1.1	nd	nd	2.4	0.3	0.9
22:6	8.0	10.1	0.5	2.4	6.7	1.8	5.7
Double bond index[c]	5.0	4.8	4.3	4.2	6.9	4.4	4.4

[a]Values represent the average of 3 determinations, using total lipids from pooled livers of 5 rats. Deviations were ±2 mol %. Traces (<0.5%) of 12:0, 12:1, 14:1 and 16:2 acids were present in most samples.

[b]Not detected.

[c]Ratio of moles double bonds:moles saturated fatty acids.

manipulation produces marked alterations in desaturase activities of rat liver microsomes with no concomitant changes in acyltransferase activities. These data suggest that regulation of the desaturases is independent of that of the transacylases and that desaturation of eicosatrienoyl-phosphatidylcholine is regulated at the level of the desaturase itself and not by availability of the phospholipid substrate.

The data also suggest that the activity of the membrane-bound acyltransferase is less sensitive to changes in the fluidity of the microsomal membranes than is that of the desaturases. It was shown previously that the increased Δ9- and Δ5-desaturase activities in membranes of animals starved and refed a fat-free diet could be correlated with decreased fluidity of membrane phospholipids as determined by fatty acid unsaturation and fluorescence polarization of parinaric acid probes (13,14). It was also shown that membranes of starved animals that have decreased Δ9- and no detectable Δ5- desaturase activities have increased fluidity of membrane phospholipids compared to that of controls (13). However, no differences were observed in acyltransferase activity in the membranes that were previously shown to have either increased or decreased fluidity of membrane phospholipids compared to the control membranes.

In addition to the effect of changes in the membrane phospholipid fluidity on desaturase activities reported earlier (10-14), other factors also appear to regulate the activity of these enzymes. For instance, the changes in desaturase activities observed on feeding diets supplemented with either corn oil or coconut oil could not be explained by changes in membrane fluidity, since the fluidity as determined by double bond index did not differ for the two diets. It may be that in these long-term dietary studies, as in those reported previously (26), steady-state membrane conditions had been reestablished. Further studies including the effect of inhibitors of protein synthesis and physical measurements of membrane fluidity will be needed to determine the reason for the observed changes in desaturase activities in these animals.

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REFERENCES

1. Uchiyama, M., Nakagawa, M., and Okui, S. (1967) J. Biochem. (Tokyo) 62, 1-6.
2. Jeffcoat, R., Brawn, P.R., and James, A.T. (1976) Biochim. Biophys. Acta 431, 33-44.
3. Holloway, C.T., and Holloway, P.W. (1975) Arch. Biochem. Biophys. 167, 496-504.
4. Martin, C.E., Hiramitsu, K., Kitajima, Y., Nozawa,

Y., Skriver, L., and Thompson, G.A. (1976) Biochemistry 15, 5218-5227.
5. Oshino, N., and Sato, R. (1972) Arch. Biochem. Biophys. 149, 369-377.
6. Strittmatter, P., Spratz, L., Corcoran, D., Rogers, M.J., Setlow, B., and Redline, R. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 4565-4569.
7. Prasad, M.R., and Joshi, V.C. (1979) J. Biol. Chem. 254, 6362-6369.
8. Lippiello, P.M., Holloway, C.T., Garfield, S.A., and Holloway, P.W. (1979) J. Biol. Chem. 254, 2004-2009.
9. Enoch, H.G., Catala, A., and Strittmatter, P. (1976) J. Biol. Chem. 251, 5095-5103.
10. Pugh, E.L., and Kates, M. (1977) J. Biol. Chem. 252, 68-73.
11. Pugh, E.L., and Kates, M. (1979) Lipids 14, 159-165.
12. Kates, M., and Pugh, E.L. (1980) in Membrane Fluidity: Biophysical Techniques and Cellular Regulation (Kates, M. and Kuksis, A., eds.) pp. 153-170, Humana Press, Clifton, NJ.
13. Pugh, E.L., Kates, M., and Szabo, A.G. (1980) Can. J. Biochem. 58, 952-958.
14. Pugh, E.L., Kates, M., and Szabo, A.G. (1982) Chem. Phys. Lipids 30, 55-69.
15. Ellingson, J.S., Hill, E.E., and Lands, W.E.M. (1970) Biochim. Biophys. Acta 196, 176-192.
16. Lapidot, Y., Rappaport, S., and Wolman, Y. (1967) J. Lipid Res. 8, 142-145.
17. Al-Arif, A., and Blecher, M. (1969) J. Lipid Res. 10, 344-345.
18. McKean, M.L., Smith, J.B., and Silver, M.J. (1982) J. Biol. Chem. 257, 11278-11283.
19. Seubert, W. (1960) Biochem. Prep. 7, 80-83.
20. Lands, W.E.M., and Hart, P. (1965) J. Biol. Chem. 240, 1905-1911.
21. Ellman, G.L. (1959) Arch. Biochem. Biophys. 82, 70-77.
22. Bligh, E.C., and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911-919.
23. Pugh, E.L., and Kates, M. (1975) Biochim. Biophys. Acta 380, 422-453.
24. Lowry, O.H., Rosebrough, M.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
25. Carroll, K.K. (1961) Nature (London) 191, 377-378.
26. Holloway, C.T., and Holloway, P.W. (1977) Lipids 12, 1025-1031.

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COMMUNICATIONS

High Field ^{13}C Nuclear Magnetic Resonance Spectrum of the Olefinic Carbons of the Triglycerides of Palm Oil

SOON NG*, *Department of Chemistry, University of Malaya, Kuala Lumpur, Malaysia*

ABSTRACT

In the triglycerides of palm oil (or any vegetable oil or fat), an unsaturated fatty acid (oleic or linoleic) attached to the 2-glycerol carbon can be distinguished from one attached to the 1- or 3-glycerol carbon by high (or medium) field ^{13}C nuclear magnetic resonance (NMR) analysis of the olefinic carbons. The chemical shift difference of the olefinic carbons in the fatty acid chain is characteristic of the chain's glycerol position and can therefore be used to identify the glycerol position of an unsaturated fatty acid in the triglyceride.
Lipids 19:56-59, 1984.

INTRODUCTION

The application of ^{13}C nuclear magnetic resonance (NMR) spectroscopy in quantitative analysis of the fatty acid composition of vegetable oils and fats has been developed by Shoolery (1). Structural identification of lipids using ^{13}C NMR was reviewed by Klein and Kemp (2). Recently we have shown that, in triglycerides of vegetable oils, the nature of the fatty acids (whether saturated, monoene or diene), attached to the 1,3- and 2-glycerol carbons, can be distinguished by the ^{13}C NMR spectrum of the carbonyl carbons of the fatty acids (3). We have also shown that the fatty acid composition of palm oil in terms of mole fractions of saturated, oleic and linoleic acids can be obtained expediently by ^{13}C NMR analysis (4). In this paper, we show that the high field ^{13}C NMR spectrum of the olefinic carbons of fatty acids in the triglycerides of palm oil (or any vegetable oil or fat) can distinguish between the unsaturated chains attached to the 1- and/or 3-glycerol carbons and those attached to the 2-glycerol carbon.

EXPERIMENTAL

The sample of palm oil used in this study was obtained from a local palm oil mill and was homogenized before use. Corn oil was obtained commercially as "Mazola" corn oil. The sample of cocoa butter was extracted from cocoa beans grown in Malaysia. Triolein was obtained from Sigma Chemical Co., St. Louis, MO.

The high field ^{13}C NMR spectra described in this work were recorded on the JEOL GX-400 spectrometer operating at 100.4 MHz in the Fourier transform mode as mentioned in the Acknowledgments. The low field ^{13}C NMR spectra were recorded on the JEOL FX-100 spectrometer operating at 25.05 MHz in the Fourier transform mode in this laboratory. The experimental details are given in the figure captions.

RESULTS AND DISCUSSION

Palm oil is composed of triglycerides of fatty acids which are mainly palmitic, oleic, linoleic and stearic (5). The low field (25 MHz or less) proton noise decoupled ^{13}C NMR spectrum of the olefinic carbons of the triglycerides of palm oil in $CDCl_3$ solution (concentration 1:3, v/v) at 28 C, shows 2 peaks at δ 130.04 and 129.72 for C-10 and C-9, respectively, of the oleic chain, and 4 peaks of lower intensity at δ 130.21, 129.98, 128.16 and 127.98 for C-13, C-9, C-10 and C-12, respectively, of the linoleic chain. The C-9 peak of the linoleic chain appears as a shoulder on the low frequency side of the C-10 peak of the oleic chain. In the low field spectrum, these peaks are slightly broadened and in favorable cases, under conditions of high resolution at 25 MHz, the oleic C-9 peak may be partially split and a shoulder may be observed on the linoleic C-10 peak. However, at medium or high field, each of these peaks is resolved into a pair of peaks of unequal intensity, with peak separations varying from 0.026 to 0.008 ppm, as shown in Figure 1.

In Figure 1, in each pair of closely spaced peaks, the higher intensity peak is assigned to the olefinic carbon of the fatty acid chain attached to the 2-glycerol carbon (designated as 2-chain), and the lower intensity peak to the

*To whom correspondence should be addressed.

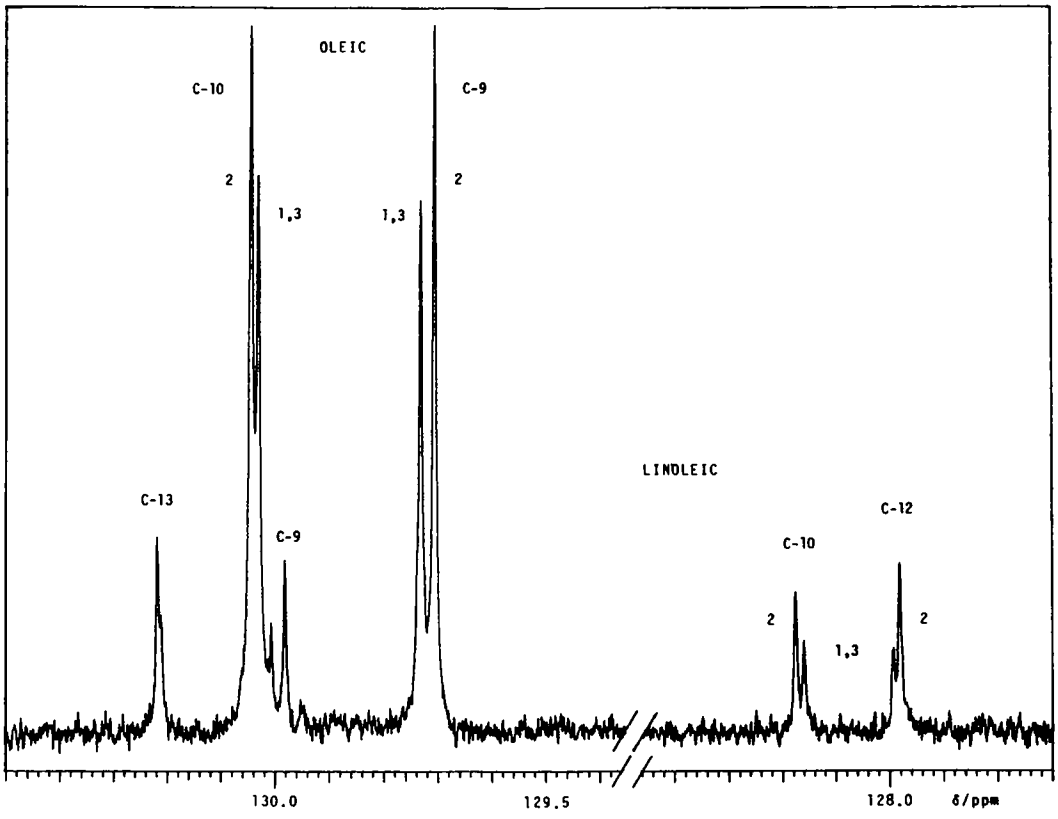

FIG. 1. High field (100.4 MHz) proton noise decoupled ^{13}C NMR spectrum of the olefinic carbons of the triglycerides of palm oil in chloroform-*d* solution (concentration 1:3 v/v) at 27 C. The assignment of the peaks is shown. The higher intensity peak (labeled 2) in each closely spaced pair belongs to the olefinic carbon of the fatty acid chain attached to the 2-glycerol carbon; the lower intensity peak (labeled 1,3) pertains to the chain attached to the 1- or 3-glycerol carbon. The chemical shifts of the peaks are (in order of the closely spaced pairs): δ 130.22, 130.21; 130.05, 130.03; 130.01, 129.98; 129.73, 129.71; 128.17, 128.16; and 127.99, 127.98. The spectrum was obtained with the following parameters: data points 16 K, spectral width 350 Hz, acquisition time 15.71 sec, pulse delay 2.0 sec, and pulse duration 9.0 μsec (for 10 mm od sample tube).

same olefinic carbon of the chain that is attached to the 1- or 3-glycerol carbon (designated as 1,3-chains). This assignment is verified by examining the high field spectra of triolein and corn oil. The major fatty acid component of corn oil is linoleic acid with the larger fraction of it attached to the 1,3-glycerol carbons (3). Thus, a high (or medium) field ^{13}C NMR spectrum can distinguish the olefinic carbons of the 2-chain from those of the 1,3-chains. Integration of the pair of peaks for C-9 of the oleic chain and of the pair of peaks for C-10 of the linoleic chain, in a spectrum obtained under suitable conditions of gated decoupling (suppressed nuclear Overhauser effect), should permit quantitative analysis of the unsaturated chains attached to the 2 glycerol positions (1,4,6).

In Figure 1, the chemical shift between a pair of peaks becomes smaller for the olefinic carbon nearer to the methyl end of the fatty acid chain; i.e., in the oleic chain, the magnitude of the peak separation is in the order: C-9 > C-10, and in the linoleic chain the order is: C-9 > C-10 > C-12 > C-13. Also, in the oleic chain, the peak for C-9 in the case of the 2-chain appears at lower frequency than in the case of the 1,3-chains; the reverse order holds for C-10. This high/low frequency alternation in the peak positions also occurs among the olefinic carbons of the linoleic chain. In the oleic chain, the chemical shift ($\Delta\delta$) between

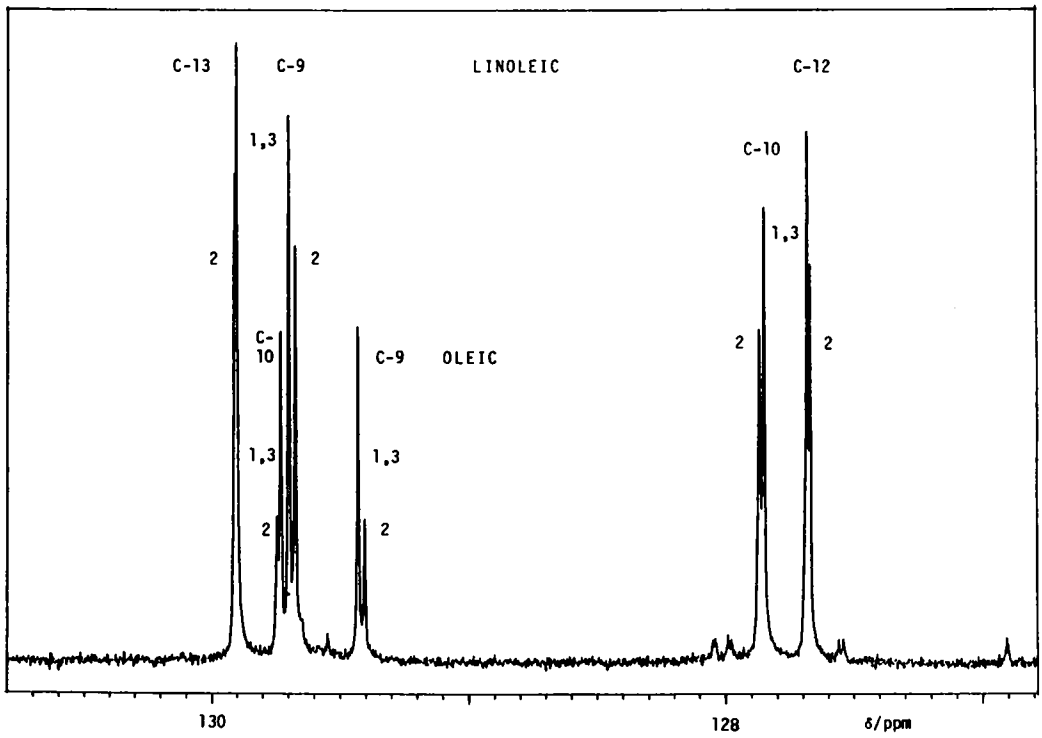

FIG. 2. High field (100.4 MHz) proton noise decoupled ^{13}C NMR spectrum of the olefinic carbons of the triglycerides of corn oil in chloroform-*d* solution (concentration 1:7, v/v) at 27 C. The spectrum is assigned as in Figure 1. Note the much higher intensity peaks for linoleic acid than for oleic acid. In each closely spaced pair, the higher intensity peak (labeled 1,3) pertains to the fatty acid attached to the 1- or 3-glycerol carbon and the lower intensity peak (labeled 2) pertains to that attached to the 2-glycerol carbon. The chemical shifts of the peaks are (in order of the closely spaced pairs): δ 129.92, 129.91; 129.75, 129.74; 129.70, 129.68; 129.44, 129.41; 127.87, 127.85; and 127.69, 127.68. The spectrum was expanded from one obtained with the following parameters: data points 64 K, spectral width 6002.4 Hz, acquisition time 2.73 sec, pulse delay 0.3 sec, and pulse duration 10 μsec (for 5 mm od sample tube in 10 mm probe). The tiny peaks present in the spectrum belong to trace amounts of unidentified components.

C-10 and C-9 is 0.34 ppm in the case of the 2-chain and 0.30 ppm in the case of the 1.3-chains. In the linoleic chain for the C-13–C-9 group of peaks, $\Delta\delta$ = 0.24 and 0.20 ppm in the 2-chain and 1,3-chains, respectively; for the C-10–C-12 group, $\Delta\delta$ = 0.19 and 0.17 ppm in the 2-chain and 1,3-chains, respectively. Consequently, in triglycerides in which unsaturated fatty acid chains are attached to only one of the 2 sets of glycerol carbons, the chemical shift differences given above may be used to determine the site of attachment of the chains. This is carried out for cocoa butter (see below).

The spectrum in Figure 2 for corn oil resembles that for palm oil in Figure 1, except that in corn oil linoleic acid is more abundant than oleic acid. The higher intensity peak in the pair for each olefinic carbon shows that more of both the linoleic and oleic acids are attached to the 1,3-glycerol carbons than to the 2-glycerol carbon (ratio ca. 1.3:1.0 for linoleic acid and ca. 2.5:1.0 for oleic acid).

Although the spectrum in Figure 3 for cocoa butter was obtained at 25.05 MHz, the sharpness of the peaks clearly indicates that they are singlets. For the 2 oleic acid olefinic carbons, the chemical shift difference ($\Delta\delta$) is 0.34 ppm, and for linoleic acid $\Delta\delta$ = 0.24 and 0.20 ppm for the C-13–C-9 and C-10–C-12 pairs, respectively. Comparing with the data deduced for the triglycerides of palm oil, these $\Delta\delta$ values show that the oleic and linoleic acids in the cocoa butter triglycerides are attached predominantly to the 2-glycerol carbon. It is known that the predominant triglyceride in cocoa butter is *sn*-palmito-oleo-stearin (7).

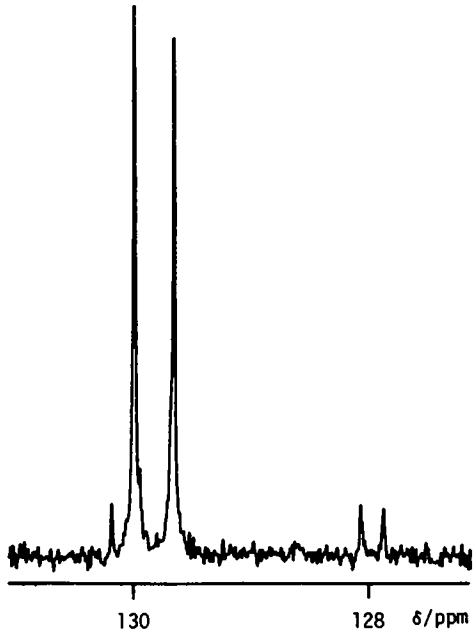

FIG. 3. Proton noise decoupled ^{13}C NMR spectrum at 25.05 MHz of the olefinic carbons of the triglycerides of cocoa butter in chloroform-*d* solution (concentration 3 mol %) at 28 C. The high intensity peaks at δ 130.01 and 129.67 belong to C-10 and C-9, respectively, of the oleic acid; the low intensity peaks at δ 130.19, 129.95, 128.09 and 127.89 belong to C-13, C-9, C-10 and C-12, respectively, of the linoleic acid in cocoa butter. The spectrum was obtained with the following parameters: data points 8 K, spectral width 400 Hz, acquisition time 2.72 sec, pulse delay 0.28 sec, and pulse angle 70°.

The results described here for olefinic carbons supplement those for carbonyl carbons in triglycerides of fatty acids reported earlier (3). The advantages of the olefinic carbons, in respect of experimental time, are their shorter spin, lattice relaxation times and larger nuclear Overhauser effect.

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REFERENCES

1. Shoolery, J.N. (1977) Prog. Nucl. Magn. Spectrosc. 11, 79-93.
2. Klein, R.A., and Kemp, P. (1977) Methods Membr. Biol. 8, 51-217.
3. Ng, S. (1983) J. Chem. Soc., Chem. Commum. 179-180.
4. Ng, S., and Ng, W.L. (1983) J. Am. Oil Chem. Soc. 60, 1266-1268.
5. Gunstone, F.D. (1967) Fatty Acids and Their Glycerides, p. 156, Chapman and Hall, London.
6. Tulloch, A.P. (1982) Lipids 17, 544-550.
7. Tsai, C.H., and Kinsella, J.E. (1982) Lipids 17, 848-852.

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Lipids of Liver, Kidney, Spleen and Muscle in a Case of Generalized Deficiency of Cytochrome b_5 Reductase in Congenital Methemoglobinemia with Mental Retardation

HARUKO HIRONO[1], *University of Göteborg, Department of Neurochemistry, Psychiatric Research Centre, Sweden*

ABSTRACT

The lipid compositions of liver, kidney, spleen and muscle in a case of generalized deficiency of cytochrome b_5 reductase in congenital methemoglobinemia with mental retardation were analyzed and compared with age-matched controls. The contents of cholesterol and phospholipids (μmol/g) in each organ were within the normal range. Diminished proportions of unsaturated fatty acids were observed in ethanolamine phosphoglycerides (EPG) of the liver. The proportion of linoleic acid decreased to less than half of the normal level in EPG of liver, kidney and spleen.

Lipids 19:48-55, 1984.

INTRODUCTION

As reported previously, there was a marked decrease in cerebrosides in white matter of the brain in a patient of generalized deficiency of cytochrome b_5 reductase in congenital methemoglobinemia with mental retardation (1). Lipid analysis also disclosed a lower level of linoleic acid and a higher level of palmitic acid in adipose tissue compared with the healthy control, showing that the patient continued to be in an underdeveloped condition (2).

In this communication, data will be presented on the lipids in liver, kidney, spleen, muscle and adrenals in the same patient.

MATERIALS AND METHODS

Autopsy materials and analytical procedure were described earlier (1,2). The samples from normals were analyzed in the same laboratory using techniques identical to those used in the analysis of the patient's samples.

RESULTS AND DISCUSSION

Table 1 shows the contents of cholesterol and lipid phosphorus (μmol/g) in liver, kidney, spleen, muscle and adrenals in the present case. There was no reduction in weight of the tissue organs. Lipid values were all within normal range, in contrast to a marked change in myelin lipids. Table 2 shows the fatty acid compositions of ethanolamine phosphoglycerides (EPG) and choline phosphoglycerides (CPG) in liver. The proportion of linoleic acid decreased to half of the normal level in EPG. Diminished proportions of unsaturated fatty acids were observed only in EPG. There were no changes in arachidonic acid contents in either EPG or CPG. The fatty acid pattern of CPG was similar to that in normals, except for slightly higher palmitic acid and lower stearic acid values.

Table 3 shows the fatty acid composition of EPG in kidney, spleen, muscle and adrenals. The proportion of linoleic acid decreased considerably, and that of arachidonic acid increased in kindney and spleen. The level of linoleic acid was normal, and arachidonic acid decreased in muscle EPG. Elevated levels of unsaturated fatty acids were observed in kidney, spleen and muscle EPG. Table 4 shows the fatty acid composition of CPG in these organs. An increased proportion of arachidonic acid in spleen and a slight decrease of linoleic acid in kidney and spleen were observed. Kidney CPG and liver CPG showed the same tendencies: higher palmitic acid and lower stearic acid levels, with no significant changes in arachidonic acid and unsaturated fatty acid levels. The proportion of linoleic acid decreased and that of arachidonic acid was unchanged in

TABLE 1

Lipid Distribution in Liver, Kidney, Spleen, Muscle and Adrenals from a Patient with Generalized Deficiency of Cytochrome b_5 Reductase

	Cholesterol (μmol/g)	Lipid phosphorus (μmol/g)
Liver	12.87	42.25
Kidney	5.61	26.68
Spleen	12.63	19.72
Muscle	1.82	15.64
Adrenals	37.75	19.44

[1]Present address: College of Medical Sciences, Tohoku University, Seiryo machi 2-1, Sendai 980, Japan.

TABLE 2

Fatty Acid Composition of EPG and CPG in Liver from a Patient with Generalized Deficiency of Cytochrome b_5 Reductase and from Normals

	Ethanolamine phosphoglycerides			Choline phosphoglycerides		
	Patient (33m)	Normal (36m)	Normal (18m)	Patient (33m)	Normal (36m)	Normal (18m)
16:0	22.0	19.2	19.0	37.5	28.4	28.9
16:1	0.8	1.2	2.0	3.2	2.8	3.1
18:0	32.0	27.3	24.2	9.7	19.4	19.7
18:1	11.7	8.5	12.4	22.2	19.2	20.1
18:2(n-6)	5.0	11.1	11.2	10.7	12.8	12.5
18:3(n-3)	1.1	0.7	1.5	0.9	0.7	1.2
20:3(n-9)			0.2		0.2	0.5
20:3(n-6)	1.5	2.7	1.2	2.1	3.1	1.9
20:4(n-6)	14.5	13.9	16.1	8.2	8.3	8.2
20:5(n-3)		0.4	1.0		0.4	0.3
22:4(n-6)	0.7	1.9	1.1	0.3	0.3	0.3
22:5(n-6)	1.2	0.9	0.7	0.4	0.3	0.3
22:5(n-3)	2.6	1.2	2.4	1.3	0.7	0.9
22:6(n-3)	9.7	11.1	7.2	3.4	3.6	2.3
Unsaturated	46.0	53.5	56.8	52.8	52.2	51.4

Values are molar percentages of fatty acid methyl esters.

TABLE 3

Fatty Acid Composition of EPG in Kidney, Spleen, Muscle and Adrenals from a Patient with Generalized Deficiency of Cytochrome b_5 Reductase and from Normals

	Kidney		Spleen		Muscle		Adrenals
	Patient (2y9m)	Normal (3y)	Patient (2y9m)	Normal (3y)	Patient (2y9m)	Normal (5-55y)[a]	Patient (2y9m)
16:0	12.7	15.4	4.9	7.4	3.7	2.4	13.4
16:1	1.0	1.2	0.5	1.0	0.9	0.5	1.5
18:0	16.5	19.3	16.4	19.0	21.5	29.8	23.3
18:1	20.2	20.4	13.2	18.0	12.4	7.5	22.8
18:2(n-6)	4.5	9.7	1.7	4.7	20.2	19.6	7.3
18:3(n-3)	1.0	0.9	0.8	1.4	1.3		1.2
20:3(n-9)		0.1		0.1			
20:3(n-6)	1.1	1.7	2.2	2.2	3.6	1.7	3.1
20:4(n-6)	37.4	26.0	37.0	29.5	25.8	31.5	20.6
20:5(n-3)		1.0		0.2			
22:4(n-6)	1.4	1.2	8.5	7.5	2.3		3.3
22:5(n-6)	0.6		1.5	0.7	1.4		1.6
22:5(n-3)	1.1	0.6	7.3	3.9	3.9		1.6
22:6(n-3)	2.6	2.6	6.1	4.5	3.1	6.9	1.5
Unsaturated	70.8	65.3	78.7	73.6	74.8	67.8	

Values are molar percentage of fatty acid methyl esters.
[a]Reference 3.

muscle CPG. The opposite effects were observed in muscle EPG, which showed a decrease in arachidonic acid and no change in linoleic acid.

A marked reduction of linoleic acid to less than half of the normal level was observed in adipose tissue triglyceride as well as in EPG of liver, kidney and spleen. The change in fatty acid composition, showing a lower level of linoleic acid and a normal level of arachidonic acid, was common in liver EPG and in CPG of kidney and muscle. Since a pronounced increase in concentration of linoleic acid in CPG with age takes place in human skeletal muscle during the early period of growth (3,4), the results obtained in muscle lipid show that the patient was in an underdeveloped state. There was no change in arachidonic acid level in muscle CPG. The arachidonic acid level is

TABLE 4

Fatty Acid Composition of CPG in Kidney, Spleen, Muscle and Adrenals from a Patient with Generalized Deficiency of Cytochrome b_5 Reductase and from Normals

	Kidney		Spleen		Muscle		Adrenals
	Patient (2y9m)	Normal (3y)	Patient (2y9m)	Normal (3y)	Patient (2y9m)	Normal (5-55y)[a]	Patient (2y9m)
16:0	35.5	29.3	44.6	44.3	29.5	29.8	30.9
16:1	2.9	2.0	3.0	2.9	4.5	2.2	3.4
18:0	9.3	17.1	11.5	15.3	7.5	9.4	13.3
18:1	29.7	24.9	19.7	19.1	21.2	14.0	25.8
18:2(n-6)	9.3	14.3	3.8	5.1	29.1	37.8	9.2
18:3(n-3)	0.9	0.8	0.8	1.2	0.6		1.0
20:3(n-9)				0.1			
20:3(n-6)	1.8	2.7	2.0	1.8	1.5	1.1	3.7
20:4(n-6)	9.0	7.6	1.9	6.2	4.4	4.8	9.8
20:5(n-3)		0.2					
22:4(n-6)	0.4		1.2	1.7	0.4		1.0
22:5(n-6)				0.2	0.1		0.2
22:5(n-3)	0.7	0.2	1.1	0.9	0.8		0.9
22:6(n-3)	0.5	0.9	0.5	1.2	0.3	0.9	0.5
Unsaturated	55.2	53.6	43.9	40.4	63.0	60.8	

Values are molar percentages of fatty acid methyl esters.
[a]Reference 3.

influenced more markedly by the dietary level of essential fatty acids than the level of linoleic acid in essential fatty acid deficient rats (5). The change in fatty acid composition in the present case, therefore, cannot be explained simply by a deficiency in nutritional supply of essential fatty acids.

Some of the circulating essential fatty acids will be incorporated in the lipids of cellular membranes, so that there might be some correlation between membrane transport and disorder of fatty acid metabolism in kidney and spleen as well as in brain. There was only a 10% reduction of unsaturated fatty acids in liver EPG among organ lipid fractions. Brain sphingolipid fractions in the patient also showed a 10% reduction of monoenes. The influence of generalized deficiency of cytochrome b_5 reductase was unexpectedly slight, if we consider that the enzyme is involved in desaturation of fatty acids (6-8). There is probably a bypass for desaturation of fatty acids. Generalized deficiency of this enzyme in the present case did not influence the desaturation or chain elongation needed for the synthesis of 20:4 (n-6) or 22:6(n-3). Unlike the case of essential fatty acid deficiency, in which the proportions of the linolenic acid series were reduced more than those of the linoleic acid series (9), there was no change in 22:6(n-3), which always constituted more than two-thirds of this series in liver phosphoglycerides.

Summarizing all the results, there was only a 10% reduction in monoenes of brain sphingolipids and in unsaturated fatty acids in liver EPG and adipose tissue. The proportion of linoleic acid decrease to less than half of the normal in EPG of liver, kidney, spleen, and in adipose triglyceride. The most severe influence of this enzyme deficiency appeared in a quantitative reduction of cerebroside content to less than half of the normal level. Generalized deficiency of cytochrome b_5 reductase might have influenced reduction of desaturation and hydroxylation which led to a reduced nervonic acid level and to a reduced amount of hydroxy fatty acids, since this enzyme system is involved in desaturation of fatty acid and in hydroxylation (10). The reduction of cerebrosides might have caused a decrease in myelination, resulting in mental retardation.

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REFERENCES

1. Hirono, H. (1980) Lipids 15, 272-275.
2. Hirono, H. (1983) Tohoku J. Exp. Med. 140, 391-394.

3. Bruce, A. (1974) J. Lipid Res. 15, 109-113.
4. Bruce, A., and Svennerholm, L. (1971) Biochem. Biophys. Acta 239, 393-400.
5. Alling. C., Bruce, A., Karlsson, I. and Svennerholm, L. (1974) Nutr. Metabol. 16, 249-259.
6. Holloway, P.W., and Katz, J.T. (1972) Biochemistry 11, 3689-3696.
7. Lee, T., Baker, R.C., Stephens, N., and Snyder, F. (1977). Biochem. Biophys. Acta 489, 25-31.
8. Okayasu, T., Ono, T., Shinojima, K., and Imai, Y. (1977) Lipids 12, 267-271.
9. Alling, C., Bruce, A., Karlsson, I., and Svennerholm, L. (1976) Nutr. Metabol. 20, 440-451.
10. Ozols, J. (1976) Ann. Clin. Res. 8 Suppl. 17, 182-192.

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A Unique Lipid Pattern Associated with the Gall Bladder Bile of the Chick Embryo

R.C. NOBLE* and **K. CONNOR**, *Hannah Research Institute, University of Glasgow, Ayr KA6 5HL, Scotland*

ABSTRACT

A study has been made of the lipid and fatty acid composition of the gall bladder bile of the chick embryo during the last week of incubation. The lipids and their fatty acid composition showed a unique pattern when compared to other animal species. Of the total lipid present, phospholipid accounted for less than half, and there were substantial proportions of both cholesteryl ester and triglyceride. In the cholesteryl ester, the proportion of which increased significantly over the last week of incubation, there was a very high level of oleic acid. The phospholipid contained a high level of arachidonic acid. The results are discussed in relation to observations on the biliary lipids of other animal species and the major features of the lipid metabolism of the chick embryo during the last week of incubation.

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INTRODUCTION

In mammalian species, the function of the bile as a vehicle for the excretion and turnover of lipids, in particular cholesterol and phospholipids, is well decumented (1,2). Indeed, phospholipid, mainly phosphatidylcholine, and free cholesterol account for virtually all the lipid of the bile, phospholipid comprising ca. 90% of the total and free cholesterol ca. 10% (1-4). The presence of only phosphatidylcholine within the bile is difficult to reconcile with the lipid composition of the suggested source of the bile lipids, namely, the plasma membranes of the cells lining the canaliculi. The possibility of specific pools of lipids within the liver which are destined for biliary secretion has therefore been suggested. The development of the chick embryo, displaying as it does a predominance on lipid metabolism during the intense period of growth over the last week of incubation (5-7), is associated with a unique pattern of liver lipid composition (6-8). A progressive accumulation of esterified cholesterol occurs in the liver such that just before hatching, cholesteryl esters account for up to 80% of the total lipid present and 30% of the total liver dry matter. Furthermore, the cholesteryl esters have an extremely high content of oleic acid which accounts for 70-75% of the total fatty acids present (6,8). Under the influence of this distinctive liver lipid composition, it appeared possible that changes to accepted bile lipid patterns might also occur. The results of a study to investigate this possibility are reported here.

*To whom correspondence should be addressed.

EXPERIMENTAL

Embryos were excised at the 13th, 15th, 17th and 19th days of incubation from fertile eggs which had been obtained from a flock of 9-month old hens kept on deep litter and which had received a conventional compound diet. The gall bladders were removed as quickly as possible after death and extracted in chloroform/methanol (2:1, v/v) acidified with hydrochloric acid (9); preliminary investigations had ascertained that the lipid patterns obtained from the complete gall bladder were identical to those of the contents. Extensive care was exercised to remove any contaminating tissue from the gall bladder. To derive sufficient material for representative analysis, it was necessary to pool the gall bladders obtained from a minimum of 8 embryos for each determination. The major neutral and phospholipid fractions were separated by established thin layer chromatographic techniques with known standards, as described in detail elsewhere (10). Quantification of the major lipid classes following their separation by thin layer chromatography was by the method described by Shand and Noble (11), involving charring and densitometry using a liquid scintillation counter. The methyl ester derivatives of the major fatty acids contained in the lipid classes were prepared by transmethylation by refluxing in the presence of dry methanolic hydrochloric acid (10) and were quantified by gas liquid chromatography on packed columns of 15% EGSS-X on Gas-Chrom P (180 C; carrier gas flow, 60 ml/min). For comparative purposes, similar analyses were also conducted on gall bladder bile of the rabbit and the sheep.

TABLE 1

The Lipid Compositions (Weight Percentage of Total Lipid) of Gall Bladder Bile from the Chick Embryo, Rabbit and Sheep[a]

	Chick embryo					
	Day 13	Day 15	Day 17	Day 19	Rabbit	Sheep
Cholesteryl ester	16.9 ± 1.25	19.2 ± 1.16	21.5 ± 0.46	29.6[d] ± 0.13	tr	tr
Triglyceride	18.9 ± 1.04	25.3 ± 2.12	20.5 ± 1.07	12.1[b] ± 1.93	tr	tr
Free fatty acid	tr	tr	tr	tr	tr	tr
Free cholesterol	17.3 ± 0.52	14.6 ± 1.27	17.3 ± 0.47	18.1 ± 0.34	12.3 ± 0.28	5.97 ± 0.43
Phospholipid	46.9 ± 1.78	40.8 ± 2.05	40.7 ± 1.50	40.3[b] ± 2.30	87.5 ± 1.01	93.9 ± 0.58

[a]Each result is the mean ± SE of 4 observations.
[b,c,d]Significantly different from result at day 13 of incubation at $P<0.05$, $P<0.01$, $P<0.001$, respectively.
tr = trace (<0.5%).

TABLE 2

The Fatty Acid Compositions (Major Fatty Acids, Weight Percentage of Total) of the Phospholipids in Gall Bladder Bile from the Chick Embryo, Rabbit and Sheep[a]

	Chick embryo					
	Day 13	Day 15	Day 17	Day 19	Rabbit	Sheep
Palmitic	33.7 ± 0.63	18.9 ± 4.37	30.5 ± 1.57	22.4[b] ± 3.48	29.5 ± 3.09	35.5 ± 1.11
Palmitoleic	2.11 ± 0.21	tr	tr	tr	3.4 ± 0.54	1.8 ± 0.15
Stearic	19.4 ± 0.39	19.9 ± 1.98	21.3 ± 1.17	24.9[d] ± 0.32	14.0 ± 0.79	16.9 ± 0.58
Oleic	25.7 ± 2.89	24.9 ± 0.16	18.4 ± 1.71	12.7[c] ± 1.01	20.8 ± 1.10	30.3 ± 0.25
Linoleic	6.05 ± 1.02	9.85 ± 2.19	14.3 ± 0.76	22.5[d] ± 1.09	23.7 ± 3.22	8.76 ± 0.37
Linolenic	tr	tr	tr	tr	1.6 ± 0.27	1.21 ± 0.07
Arachidonic	15.7 ± 1.56	13.9 ± 4.07	15.6 ± 1.95	17.8 ± 4.52	3.18 ± 0.41	5.46 ± 0.45

[a]Each result is the mean ± SE of 4 observations.
[b,c,d]Significantly different from result at day 13 of incubation at $P<0.05$, $P<0.01$, $P<0.001$, respectively.
tr = trace (<0.5%).

RESULTS

The distributions (weight percentages of the total lipid) of the major lipid fractions present in the bile of the chick embryo during the last week of incubation and the biles from the rabbit and sheep are given in Table 1. As can be seen, in the bile of the rabbit and sheep phospholipid was by far the major lipid component present, with free cholesterol the only other lipid of significant proportion. In contrast, phospholipid accounted for less than half of the total lipid present in the chick embryo bile over the last week of incubation. As well as free cholesterol, substantial amounts of both cholesteryl ester (up to 30% of total lipid) and triglyceride (up to 25% of total lipid) were also present. During the last week of incubation, the proportion of the cholesteryl esters increased significantly while there was a concomitant reduction in the proportion of triglyceride. The proportions of free cholesterol and phospholipid remained virtually unchanged over the last week of incubation. In all the biles, phosphatidylcholine constituted the principal component (>95%) of the phospholipid fraction.

Table 2 shows the concentrations (weight percentages of the total) of the major long-chain fatty acids associated with the phospholipid fraction of the biles. In each case, palmitic and stearic acids accounted for ca. 50% of the total fatty acids present. In contrast to the rabbit bile in which there was a substantial level of linoleic acid present and a lower proportion of oleic acid, there was a relatively low level of linoleic acid in the sheep bile. The phospholipid of the chick embryo bile also contained a high proportion of linoleic acid but, unlike that of the rabbit and sheep, there was also a high proportion of arachidonic acid. During the last week of incubation, the proportion of oleic acid in the biliary phospho-

TABLE 3

The Fatty Acid Compositions (Major Fatty Acids, Weight Percentage of Tatal) of the Cholesteryl Esters of Gall Bladder Bile from the Chick Embryo[a]

	Day 13	Day 15	Day 17	Day 19
Palmitic	29.7 ± 1.26	17.2 ± 2.30	16.2 ± 1.26	4.73 ± 0.14[d]
Palmitoleic	3.70 ± 0.62	1.59 ± 0.38	1.64 ± 0.11	tr[d]
Stearic	17.5 ± 0.63	15.2 ± 2.68	11.3 ± 1.80	5.09 ± 0.19[d]
Oleic	38.5 ± 3.77	61.2 ± 2.05	64.6 ± 3.23	75.3 ± 0.58[d]
Linoleic	9.00 ± 1.72	5.83 ± 1.60	7.88 ± 0.98	14.7 ± 0.52[c]

[a]Each result is the mean ± SE of 4 observations.
[b,c,d]Significantly different from result at day 13 of incubation at $P<0.05$, $P<0.01$, $P<0.001$, respectively.
tr = trace (<0.5%).

TABLE 4

The Fatty Acid Compositions (Major Fatty Acids, Weight Percentage of Tatal) of the Triglycerides of Gall Bladder Bile from the Chick Embryo[a]

	Day 13	Day 15	Day 17	Day 19
Palmitic	29.8 ± 2.04	26.0 ± 0.61	32.3 ± 2.49	28.0 ± 2.67
Palmitoleic	2.00 ± 0.41	2.58 ± 0.66	tr	tr
Stearic	14.7 ± 0.54	14.6 ± 0.85	18.5 ± 0.99	19.1 ± 1.55[c]
Oleic	45.2 ± 3.55	50.1 ± 0.92	43.7 ± 1.24	42.3 ± 3.14
Linoleic	7.91 ± 0.41	6.68 ± 0.57	5.53 ± 0.66	10.6 ± 2.31[b]

[a]Each result is the mean ± SE of 4 observations.
[b,c,d]Significantly different from result at day 13 of incubation at $P<0.05$, $P<0.01$, $P<0.001$, respectively.
tr = trace (<0.5%).

lipid of the chick embryo diminished significantly and the proportion of linoleic acid increased.

The fatty acid compositions (weight percentages of the total fatty acids present) in the cholesteryl ester and triglyceride fractions of the chick embryo bile during the last week of incubation are given in Tables 3 and 4, respectively. As can be seen, the cholesteryl esters associated with the bile over the last week of incubation was accounted for largely by the secretion of cholesteryl oleate, such that by the 19th day of incubation ca. 75% of the total fatty acids associated with the cholesteryl esters was oleic acid. Oleic acid was also the major fatty acid present in the triglyceride fraction, but at a level much lower than that within the cholesteryl esters.

DISCUSSION

The results obtained in the present investigations for the lipid and fatty acid compositions of rabbit and sheep bile conformed to the patterns already established for a range of other animal species (3,4,12,13). However, it is clear that the biliary lipids of the chick embryo differ quite substantially from these established patterns. The major points of difference were the presence of large quantities of both cholesteryl esters and triglycerides, fractions which are notably absent from the bile of other species, and the predominance of oleic acid within the cholesteryl ester fraction.

The appearance of substantial quantities of cholesteryl oleate in the bile of the chick embryo during the last week of incubation parallels the extensive accumulation of cholesteryl oleate within the liver of the chick embryo over this period, such that it becomes the most abundant lipid in the liver (6-8). As a result of recent investigations (14), it has become clear that the cholesteryl oleate originates from synthesis in the yolk sac membrane where it plays a vital role in the uptake and subsequent assimilation of some 6-8 g of yolk lipid by the embryo during the last week of incubation. The subsequent deposition and accumulation of the cholesteryl oleate in the liver represents the fulfillment of this role.

It is not possible to associate the presence of substantial quantities of triglyceride in the bile of the chick embryo with any comparable compositional change in the embryo liver. Certainly, the amount of triglyceride absorbed by the chick embryo during the last week of incubation is large, amounting to ca. 4 g (5,6), but triglyceride levels in the liver do not rise and by the end of incubation they account for no more than 2% of the total lipid present (6,8,15). However, recent preliminary investigations of the biliary lipid composition of the chicken (Noble and Connor, unpublished obserbations) have shown that, although there is a rapid diminution in the levels of cholesteryl ester after hatching, the triglycerides remain as an important component of the bile in both the immature and mature bird. The substantial amounts of cholesteryl ester in the bile of the embryo, therefore, may represent a transient feature imposed by an exceptional liver lipid composition and may represent a mechanism through which the hepatic accumulation of cholesteryl ester is reduced. Similarly, the presence of triglyceride in the bile in the mature bird may represent a mechanism for controlling the hepatic level of triglyceride particularly in the laying bird. Whereas in other animal species, it is probable that extensive recycling of biliary lipid occurs, either through direct reabsorption of intact phospholipids or reacylation of the lyso derivative (16,17), the fate of the biliary lipids secreted by the chick embryo remain unknown.

The presence of a high proportion of arachidonic acid in the biliary phospholipid of the chick embryo is similar to the findings for the rat, pig and dog (12,13). However, in species other than ruminants (4), linoleic acid comprises the principal biliary unsaturated fatty acid. The accumulation of high levels of arachidonic acid, in association with the phospholipid fraction, has also been shown to be a feature of lipid metabolism of the chick embryo liver during incubation (18). In the absence of substantial quantities of arachidonic acid in the lipids of the yolk (19), the presence of an active desaturase system in the embryonic tissues is implicated.

In all animal tissues so far studied, it is well known that the bile plays an important role in cholesterol excretion (1,2). From the present investigations, it appears that, as a result of unusual lipid accumulation in the liver, in the chick embryo and also probably the mature bird, bile also provides a pathway for the excretion of other lipid metabolites. The importance of such a pathway in lipid homeostasis under other abnormal lipid metabolic conditions may therefore deserve consideration.

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REFERENCES

1. Myant, N.B. (1981), in The Biology of Cholesterol and Related Steroids, 1st edn., pp. 853-887, William Heinemann Medical Books Ltd., London.
2. Portman, O.W., Osuga, T., and Tanaka, N. (1975) Adv. Lipid Res. 13, 135-194.
3. Polonowski, M., and Bourrillon, R. (1982) Bull. Soc. Chem. Biol. Paris 34, 703-712.
4. Christile, W.W. (1978) Prog. Lipid Res. 17, 111-205.
5. Romanoff, A.L. (1960) The Avian Embryo. Structural and Functional Development, McMillan Co., New York.
6. Noble, R.C., and Moore, J.H. (1964) Can. J. Biochem. 42, 1729-1741.
7. Moore, J.H., and Doran, B.M. (1962) Biochem. J. 84, 506-513.
8. Noble, R.C., and Moore, J.H. (1966) in Physiology of the Domestic Fowl (Horton-Smith, C., and Amoroso, E.C., eds.) pp. 87-102, Oliver and Boyd Ltd., Edinburgh.
9. Lough, A.K., Felinski, L., and Garton, G.A. (1962) J. Lipid Res. 3, 478-480.
10. Christie, W.W. (1982) Lipid Analysis, 2nd edn., Pergamon Press, Oxford.
11. Shand, J.H., and Noble, R.C. (1980) Anal. Biochem. 101 427-434.
12. Balint, J.A., Kyriakides, E.C., Spitzer, H.L., and Morrison, E.S. (1965) J. Lopid Res. 6, 96-99.
13. Christie, W.W. (1973) Biochim. Biophys. Acta 316, 204-211.
14. Noble, R.C., Connor, K., and Smith, W.K. (1984) Poultry Sci., (to be published Feb, 1984).
15. Noble, R.C., and Moore, J.H. (1965) Biochem. J. 95, 144-149.
16. Boucrot, P. (1972) Lipids 7, 282-288.
17. Noble, R.C. (1978) Prog. Lipid Res. 17, 55-91.
18. Noble, R.C., and Moore, J.H. (1967) Can. J. Biochem. 45, 627-639.
19. Noble, R.C., and Moore, J.H. (1967) Can. J. Biochem. 45, 949-958.

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A Nuclear Magnetic Resonance Spectroscopic Investigation of the Headgroup Motions of Lysophospholipids in Bilayers

W. WU[1], F.A. STEPHENSON', J.T. MASON and C. HUANG*, *Department of Biochemistry and Biophysics Program, University of Virginia School of Medicine, Charlottesville, VA 22908*

ABSTRACT

Fully hydrated lysophospholipids in both the gel and the liquid crystalline states have been shown to exhibit negative phosphorus chemical shift anisotropies ($\Delta\sigma$) by ^{31}P nuclear magnetic resonance (NMR). The magnitude of $\Delta\sigma$ for monoacyl lysophospholipids is found to be smaller than that of the corresponding diacyl phosphospholipids in bilayers by about a factor of two. We present evidence to suggest that the reduction in $\Delta\sigma$ can be attributed primarily to an additional motional averaging of the lysophospholipid headgroup, and the additional motion can best be explained by rapid rotational motions of the headgroup about the C(1)-C(2) glycerol bond.
Lipids 19:68-71, 1984.

^{31}P NMR spectroscopy has been recently employed to characterize the conformational and motional properties of phospholipids in bilayers and biomembranes (1,2). Most of the ^{31}P nuclear magnetic resonance (NMR) studies have been carried out with diacyl phospholipids. For instance, the ^{31}P NMR powder spectrum of diacyl phosphatidylcholine liposomes exhibits a negative chemical shift anisotropy ($\Delta\sigma$) which is characterized by a high-field peak with a broad low-field shoulder. This resonance shape arises from axial rotation of the headgroups in bilayers which results in the motional averaging of the anisotropic chemical shift tensor to a pseudoaxially symmetric one (3); the ^{31}P NMR spectrum exhibiting this resonance shape is often called a "bilayer" type spectrum. The negative chemical shift anisotropy of the axial symmetric spectrum for diacyl phosphatidylcholines in the liquid crystalline state is ca. 40-50 ppm (4), whereas in the gel state it is ca. 55-69 ppm (5). The decrease in the absolute value of $\Delta\sigma$ for diacyl phospholipids in going from the gel to the liquid-crystalline state has been attributed to the onset of an additional motion of the diacyl phospholipid molecule accompanying the gel $\rightarrow$ liquid-crystalline phase transition (6). Specifically, this additional motion is assigned to the wobbling of the headgroup with rapid rotation about the C(2)-C(3) axis of the glycerol backbone coupled with conformational transitions of the phosphocholine group (6,7).

Recently, it has been shown that fully hydrated lysophosphatidylcholines in the interdigitated gel state exhibit a "bilayer" type ^{31}P NMR spectrum (8,9). Interestingly, the chemical shift anisotropy of the lysophospholipid lamella (30-40 ppm) is significantly smaller than that of the diacyl phospholipid bilayer in the noninterdigitated gel state, indicating a considerable additional motional averaging of the phosphorus chemical shift anisotropy and/or a conformational change of the headgroup (4,9). In this communication, we present a molecular model involving the bond rotation between the C(1) and the C(2) atom of the glycerol backbone of monoacyl lysophospholipids which may explain the characteristic ^{31}P chemical shift anisotropy of monoacyl lysophospholipids in the lamella.

^{31}P NMR powder spectra of egg phosphatidylcholine dispersions and egg lysophosphatidylcholine/cholesterol (1:1 molar ratio) codispersions obtained at room temperature are shown in Figure 1. It is observed that the ^{31}P NMR spectrum of egg lysophosphatidylcholine/cholesterol dispersions has the shape of a "bilayer" type signal superimposed on an isotropic peak. This composite spectrum suggests that egg lysophosphatidylcholine and cholesterol form large bilayer structures with some residual lysophospholipid micelles and/or small mixed lipid vesicles in the 1:1 lysophosphatidylcholine/cholesterol dispersion. The observation of a "bilayer" spectrum is consistent with the conclusion drawn from X-ray diffraction and calorimetric studies on lysophosphatidylcholine/cholesterol complexes (10,11). An important feature shown in Figure 1 is that the ^{31}P

[1]Present address: Department of Biological Sciences, Carnegie-Mellon University, Pittsburg, PA 15213.

*To whom correspondence should be addressed.

chemical shift anisotropy of egg phosphatidylcholine dispersions (47 ppm) in the liquid crystalline state is considerably larger than that of egg lysophosphatidylcholine/cholesterol dispersions (25 ppm); this is also the case in the liquid crystalline state. Recently, Allegrini et al. (4) reported that the ^{31}P chemical shift anisotropy of dipalmitoylphosphatidylcholine dispersions is larger than that of 1-palmitoyllysophosphatidylcholine/palmitic acid codispersions. For instance, at a temperature (40 C) near the transition temperature, the ratio of the chemical shift anisotropy for the two systems is 1.8 (4), which is virtually identical to the relative value (1.9) observed for egg phosphatidylcholine and egg lysophosphatidylcholine/cholesterol dispersions (Fig. 1). Clearly, ^{31}P NMR spectra of various bilayer systems containing monoacyl lysophosphatidylcholines in either the gel or the liquid crystalline state display a reduced $\Delta\sigma$ in comparison with the corresponding diacyl phosphatidylcholine bilayers.

To demonstrate that the reduction in $\Delta\sigma$ for lamellar lysophosphatidylcholines is a general characteristic for all lysophospholipid molecules in bilayers, we have carried out comparative ^{31}P NMR studies on lysophosphatidylethanolamine and diacyl phosphatidylethanolamine dispersions. Figure 2 shows the ^{31}P NMR spectra of 1-palmitoyllysophosphatidylethanoamine and dipalmitoylphosphatidylethanolamine dispersions recorded at a common reduced temperature, which corresponds to 42 C below the 57 C gel lamellar → micellar transition temperature of 1-palmitoyllysophosphatidylethanolamine dispersions and also 42 C below the 63 C gel → liquid crystalline phase transition temperature of dipalmitoylphosphatidylethanolamine dispersions. Here we have clearly demonstrated that lysophosphatidylethanolamine as well as lysophosphatidylcholine molecules in excess water exhibit a "bilayer" spectrum with considerable reduction in chemical shift anisotropy compared to the corresponding diacyl phospholipid systems. In the gel state, the low-field shoulder of the "bilayer" spectrum shown in Figure 2 is too broad to be resolved from the high-field peak. The value of $\Delta\sigma$, however, can be estimated from the bandwidth at the half of the maximum peak height (12). The half-width for diacyl phosphatidylethanolamine and monoacyl lysophosphatidylethanolamine dispersions are 70 and 36 ppm, respectively. The ratio of $\Delta\sigma$ for diacyl phosphatidylethanolamine to monoacyl lysophosphatidylethanolamine dispersions recorded at a common reduced temperature in the gel state is thus 1.9.

The observation that the ratio of 1.9 for

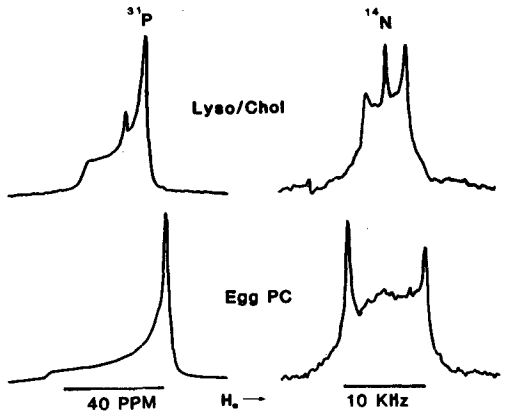

FIG. 1. 145.7 MHz ^{31}P NMR and 26.0 MHz ^{14}N NMR spectra of egg diacylphosphatidylcholine and egg lysophosphatidylcholine/cholesterol (1:1 molar ratio) dispersions taken in 10-mm tubes at room temperature on a Nicolet NICFT-1180 spectrometer. ^{31}P NMR spectra were obtained under continuous broadband proton decoupling. A sweep width of 100 kHz was employed, 8k data points were collected and 1000 scans per spectrum at a delay of 0.3 sec between pulses were applied. ^{14}N NMR spectra were obtained using a two-pulse quadrupole echo method (16). A 150-Hz line broadening was introduced during signal enhancement. Phospholipid concentrations were 200 mg/ml and a 2 ml volume of lipid sample was used. Prior to use, the lipid or lipid mixture was first lyophilized and then vortexed in distilled H_2O.

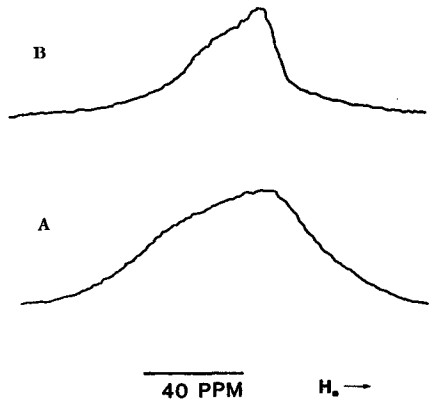

FIG. 2. 24.15 MHz ^{31}P NMR spectra of dipalmitoyl phosphatidylethanolamine (A) and 1-palmitoyllysophosphatidylethanolamine (B) dispersions taken in 10-mm tubes at 21 C and 15 C, respectively, on a Joel-FX 60Q Fourier-transform spectrometer under continuous broadband proton decoupling. A 4-kHz sweep width was employed, and 50,000 scans per spectrum averaged with 1-sec delay were applied. A line broadening of 1 Hz was introduced during signal enhancement, and 4k data points were collected. Lipid was 150 mM in 50 mM KCl.

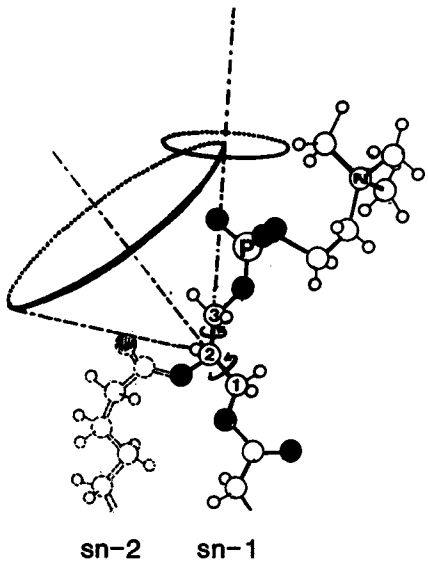

FIG. 3. A schematic diagram showing the molecular structure of diacylphosphatidylcholine and lysophosphatidylcholine. The carbon atoms in the glycerol backbone region are indicated numerically. The monoacyl lysophosphatidylcholine is taken to have a structure similar to diacyl phosphatidylcholine, except that the *sn*-2 acyl chain (the dashed structure) is replaced by a proton.

relative $\Delta\sigma$ obtained from ^{31}P NMR spectra of diacyl phosphatidylethanolamine and monoacyl lysophosphatidylethanolamine dispersions (Fig. 2) is identical to that obtained from egg phosphatidylcholine and egg lysophosphatidylcholine/cholesterol dispersions (Fig. 1) is by no means trivial.

Theoretically, the change in $\Delta\sigma$ can be attributed to conformational and/or motional differences of the diacyl and monoacyl phospholipids in bilayers with respect to the PO_4 moiety of the headgroup (13,14). Since the headgroup conformation of phosphatidylcholine and phosphatidylethanolamine molecules in bilayers are not completely identical (15), the conformational change of the headgroup which might be associated with the removal of *sn*-2 acyl chain would thus be expected to be also different for lysophosphatidylcholine and lysophosphatidylethanolamine. The fact that the magnitude and the direction of the change in $\Delta\sigma$ in going from phosphatidylcholine to lysophosphatidylcholine is identical to those in going from phosphatidylethanolamine to lysophosphatidylethanolamine strongly suggests that the conformational change, if it does play a role, cannot contribute significantly to the experimentally observed ^{31}P NMR results (Figs. 1 and 2).

To substantiate further the minimum involvement of the conformational change of headgroups in bilayers as lipids are converted from diacyl phospholipids to monoacyl lysophospholipids, ^{14}N NMR powder spectra of egg phosphatidylcholine dispersions and egg lysophosphatidylcholine/cholesterol (1:1 molar ratio) were recorded at room temperatures at a frequency of 26.0 MHz with a NICFT-1180 spectrometer following the procedure of Siminovitch et al. (16). Results from Figure 1 indicate that the ^{14}N quadrupole splitting, $\Delta\nu$, for egg phosphatidylcholine liposomes (10.6 kHz) is significantly greater than that for egg lysophosphatidylcholine/cholesterol bilayers (5.6 kHz). Interestingly, the ratio of 1.9 in $\Delta\nu$ for the two lipid systems is identical to the relative value in $\Delta\sigma$ observed for the same two lipid systems by ^{31}P NMR (Fig. 1). This same relative reduction in $\Delta\nu$ and $\Delta\sigma$ strongly suggests that the average static orientation of the headgroup of diacyl phospholipids is very similar to that of monoacyl lysophospholipids in the bilayer. High-resolution ^{1}H and ^{13}C NMR studies of phosphatidylcholines and lysophosphatidylcholines by Hauser et al. (17) also demonstrated that the preferred conformation of the phosphorylcholine moiety of the headgroup in diacyl phospholipids is very similar to that for monoacyl lysophospholipids.

If the static headgroup conformation of diacyl phospholipids in bilayers is not significantly different from that of monoacyl lysophospholipids, the observed large reduction in $|\Delta\sigma|$ must be attributed primarily to a considerable additional motional averaging of the phosphorus chemical shift anisotropy exhibited by lysophospholipids in bilayers. For lysophospholipids, the *sn*-2 acyl chain of diacyl phospholipids is substituted by a hydrogen atom. Due to the removal of the stereospecific and bulky *sn*-2 acyl chain, the C(1)-C(2) bond in the glycerol backbone for monoacyl lysophospholipid molecules can be shown, by use of CPK-space filling model, to be able to rotate freely. This rotational motion, which is fast on an NMR time scale, must contribute to the overall motion of the lysophospholipid headgroup detected by ^{31}P NMR. The phosphorus chemical shift anisotropy detected for diacyl phospholipids should thus be observed to be reduced by the same additional rotational motions about the C(1)-C(2) bond of the glycerol backbone, for the lysophospholipids, in both the gel and the liquid crystalline states. For instance, in the liquid-crystalline state, this rotational motion about the C(1)-C(2) glycerol

bond will enhance the angular amplitude of the axial rotation of the headgroup around the C(2)-C(3) bond in the glycerol backbone region (Fig. 3), resulting in additional motional averaging. Our model thus postulates that the reduction in $\Delta\sigma$ for monoacyl lysophospholipids in bilayers in both the gel and the liquid crystalline states is primarily due to the additional rapid motions at the C(1)-C(2) glycerol bond.

ACKNOWLEDGMENTS

We thank Professor R. B. Martin for helpful discussions and W. C. Hutton for assistance in obtaining some of the NMR spectra. This study was supported, in part, by Research Grant GM-17452 from the National Institute of General Medical Sciences, U.S. Public Health Service. Portions of this investigation were taken from the dissertation submitted by W. Wu in August, 1983, to the University of Virginia in partial fulfillment of the requirements for a Ph.D. degree in Biophysics.

REFERENCES

1. Seelig, J. (1978) Biochim. Biophys. Acta 515, 105-141.
2. Yeagle, P.L. (1978) Acc. Chem. Res. 11, 321-327.
3. Kohler, S.J., and Klein, M.P. (1977) Biochemistry 16, 519-526.
4. Allegrini, P.R., Scharrenburg, G.V., de Hass, G.H., and Seelig, J. (1983) Biochim. Biophys. Acta 731, 448-455.
5. Brown, M.F., and Seelig, J. (1978) Biochemistry 17, 381-384.
6. Griffin, R.G., Powers, L., and Pershan, P.S. (1978) Biochemistry 17, 2718-2722.
7. Gally, H.U., Pluschke, G., Overath, P., and Seelig, J. (1981) Biochemistry 20, 1826-1831.
8. Wu, W., Huang, C., Conley, T.G., Martin, R.B., and Levin, I.W. (1982) Biochemistry 21, 5957-5961.
9. Van Echteld, C.J.A., deKruijff, B., Mandersloot, J.G., and de Gier, J. (1981) Biochim. Biophys. Acta 649, 211-220.
10. Rand, R.P., Pangborn, W.A., Purdon, A.D., and Tinker, D.O. (1975) Can. J. Biochem. 53, 189-195.
11. Ramsammy, L.S., and Brockerhoff, H. (1982) J. Biol. Chem. 257, 3570-3574.
12. Cullis, P.R., deKruijff, B., and Richards, R.E. (1976) Biochim. Biophys. Acta 426, 433-446.
13. Thayer, A.M., and Kohler, S.J. (1981) Biochemistry 20, 6831-6834.
14. Noggle, J.H., Marecek, J.F., Mandal, S.B., van Venetie, R., Rogers, J., Jain, M.K., and Ramirez, F. (1982) Biochim. Biophys. Acta 691, 240-248.
15. Buldt, G., and Wohlgemuth, R. (1981) J. Membr. Biol. 58, 81-100.
16. Siminovitch, D.J., Rance, M., and Jeffrey, K.R. (1980) FEBS Lett. 112, 79-82.
17. Hauser, H., Guyer, W., Pascher, I., Skrabal, P., and Sundell, S. (1980) Biochemistry 19, 366-373.

[Received August 17, 1983]

ERRATUM

In the article "1-*O*-Alkyl-Linked Phosphoglycerides of Human Platelets: Distribution of Arachidonate and Other Acyl Residues in the Ether-Linked and Diacyl Species," by H. W. Mueller, A. D. Purdon, J. B. Smith and R. L. Wynkle (*Lipids* 18:814-819, 1983), Table 2 was printed missing the third figure in the second column. This table is reproduced correctly here.

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	Choline-containing phosphoglycerides (mol %) (N = 3)[a]	Ethanolamine-containing phosphoglycerides (mol %) (N = 3)[a]
1,2-Diacyl	81.8 ± 2.6	36.1 ± 0.3
1-*O*-Alkyl-2-acyl	9.7 ± 0.3	3.5 ± 0.1
1-*O*-Alk-1′-enyl-2-acyl	8.8 ± 2.4	60.4 + 0.4

[a]The data are presented as the mean ± standard deviation of 3 separate determinations done on the purified platelet PC and PE fractions.

bond will enhance the angular amplitude of the axial rotation of the headgroup around the C(2)-C(3) bond in the glycerol backbone region (Fig. 3), resulting in additional motional averaging. Our model thus postulates that the reduction in $\Delta\sigma$ for monoacyl lysophospholipids in bilayers in both the gel and the liquid crystalline states is primarily due to the additional rapid motions at the C(1)-C(2) glycerol bond.

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REFERENCES

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12. Cullis, P.R., deKruijff, B., and Richards, R.E. (1976) Biochim. Biophys. Acta 426, 433-446.
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[a]The data are presented as the mean ± standard deviation of 3 separate determinations done on the purified platelet PC and PE fractions.

Plasma and Lipoprotein Lipid Responses to Four Hypolipid Drugs

WILLIAM R. HAZZARD*[1], PATRICIA W. WAHL, CLAUDE GAGNE[2], DEBORAH APPLEBAUM–BOWDEN, G. RUSSELL WARNICK and **JOHN J. ALBERS[3]**, *Northwest Lipid Research Clinic and Departments of Medicine and Biostatistics, University of Washington, Seattle, WA 98195*

ABSTRACT

The responses of 14 hyperlipidemic subjects to 4 hypolipidemic agents were compared by measureing cholesterol and triglyceride in whole plasma, very low density lipoproteins (VLDL), low density lipoproteins (LDL), and high density lipoproteins (HDL) monthly for 2 months before and 3 months during treatment with each of 4 drugs: clofibrate, 2 g/d; colestipol, 20 g/d; para-aminosalicylic acid-ascorbate (PAS-C), 6-8 g/d; and oxandrolone, 7.5 mg/d. Lipid responses proved to be stable by the first monthly evaluation both off and on each drug. Mean adherence was high and similar for all agents (81-92% of the prescribed dose). Clofibrate was associated with significant decreases in mean plasma cholesterol (-16%, $p<.01$), plasma triglyceride (-51%, $p<.005$), VLDL-cholesterol (-61%, $p<.005$) and VLDL-triglyceride (-61%, $P<.005$), while HDL cholesterol increased (+20%, $p<.01$), and the LDL-cholesterol/HDL ratio declined (-24%, $p<.05$). Colestipol was associated with decreases in mean plasma cholesterol (-15%, $p<.01$) and LDL-cholesterol (-22%, $p<.05$), while VLDL-triglyceride increased (+41%, $p<.05$), and the LDL-cholesterol/HDL-cholesterol ratio declined (-25%, $p<.05$). PAS-C was associated with decreases in VLDL-cholesterol (-30%, $p<.05$), and VLDL-triglyceride (-29%, $p<.05$), while the LDL-cholesterol/HDL-cholesterol ratio remained unchanged. Oxandrolone was associated with increases in mean plasma cholesterol (+7%, $p<.05$), LDL-cholesterol (+45%, $p<.005$ [+25% excluding one subject who increased 298%]), and LDL-triglyceride (+24%, $p<.01$), while decreases occurred in plasma triglyceride (-31%, $p<.05$), VLDL-cholesterol (-26%, $p<.05$), VLDL-triglyceride (-42%, $p<.005$), HDL-cholesterol (-45%, $p<.005$), and HDL-triglyceride (-43%, $p<.01$). The mean LDL-cholesterol/HDL-cholesterol ratio increased by 109% ($p<.005$), reflecting the reciprocal changes in LDL and HDL. Thus, while both clofibrate and colestipol were associated with significant, equivalent reductions in theoretical atherogenic risk, oxandrolone produced a net effect that was not only adverse but 4 times that magnitude, suggesting caution in its long-term use, even for the management of hypertriglyceridemia.

Lipids 19:73-79, 1984.

INTRODUCTION

The pharmacological management of dyslipoproteinemia is complex, reflecting the numerous hypolipidemic drugs available with varying degrees of efficacy and mode of action, the heterogeneity – genetic and otherwise – of hyperlipidemic patients, the potentially confounding effects of coexisting conditions and medications that may affect blood lipid levels, coprescribed dietary intervention, and variations in patient compliance with dietary and drug regimens (including noncompliance attributable to drug side effects). To begin to lessen uncertainties in the prescription of drugs to reduce lipid levels, the present systematic evaluation of 4 such agents (clofibrate, colestipol, para-aminosalicylic acid-ascorbate [PAS-C], and oxandrolone) was undertaken with special emphasis on careful selection of subjects and their genetic characterization through family studies. A previous report has detailed the aspects of this study related to whole plasma and high density lipoprotein (HDL) cholesterol and apolipoprotein concentrations (1). The present report summarizes the cholesterol and triglyceride responses in very low density lipoproteins (VLDL) and low density lipoproteins (LDL) in addition to HDL, emphasizing the change in LDL-cholesterol/HDL-cholesterol, a widely used contemporary lipoprotein index of atherogenic risk (2).

METHODS

Subjects

Hyperlipidemic volunteers were recruited from referrals to the Northwest Lipid Research Clinic of the University of Washington. After the objectives and requirements of the study were explained in detail, informed consent was obtained. The volunteer was not told his or her

*To whom correspondence should be addressed.

[1]William R. Hazzard was an investigator of the Howard Hughes Medical Institute during the course of this study. [2]Claude Gagne was a research fellow of the Medical Research Council of Canada during the course of this study. [3]John J. Albers is an established investigator of the American Heart Association.

lipid levels until completion of the study. Nine of the 14 study participants were males. Subjects ranged in age from 28 to 66 yr (averaging 41 yr). A detailed family history, as described by Brunzell et al. (3), permitted classification of the hyperlipidemia into 1 of 4 inherited disorders in 10 of the 14 cases: 5 (including a mother and son) had familial hypercholesterolemia (FH), 3 had Type III hyperlipidemia (Broad-β disease) (BBD), and one each had familial combined hyperlipidemia (FCHL) and familial hypertriglyceridemia (FHTG). The diagnosis of BBD required the repeated demonstration of β-VLDL on agarose electrophoresis (4) of the isolated $d<1.006$ lipoproteins and a VLDL cholesterol/plasma triglyceride ratio consistently exceeding 0.30 (5). In addition, 2 of the 3 participants with this disorder have subsequently demonstrated a lack of isoapolipoproteins E_3 and E_4 (the third was not tested) (6). Family studies on the remaining 4 participants did not permit a genetic classification (UNCL). All 4 tended to have elevations of both cholesterol and triglyceride. Two of these were twins, purportedly monozygotic.

Study Design

All measurements were performed on an outpatient basis at the Clinical Research Center or the Northwest Lipid Research Center at Harborview Medical Center. These outpatient visits occurred at monthly intervals, 22 in number for subjects completing the entire 4-drug sequence. Three years were required for the total study. At the first visit, a complete cardiovascular history was obtained and a physical evaluation was performed, including resting and near-maximal exercise electrocardiography. No subject taking a drug known to affect lipid metabolism was studied. The subjects were also instructed to eat a diet moderately restricted in cholesterol (ca. 400 mg daily) and saturated fat (polyunsaturated/saturated ratio 0.6-1.0). Thereafter, the diet was reinforced at each monthly visit by an interview with a registered dietitian, with emphasis placed on consistency in the dietary pattern. No systematic alteration in diet was associated with any drug used in this study, nor did weight change significantly. Other monthly data gathered by a research nurse included a standarized medical history that included questions as to the following side effects: nausea, vomiting, bloating, abdominal cramps, diarrhea, constipation, anorexia, increased appetite, fever, headache, weakness, dizziness, drowsiness, and breast discomfort. After 3 baseline visits, the first drug was dispensed, with instructions about potential side effects, by a clinical investigator (WRH or CG) who thereafter adjusted the drug dosage as necessary to control side effects (required only for PAS-C). All unused drug portions were returned each month and adherence was calculated by a pill or packet count. Each drug, i.e. clofibrate (1 g twice daily), colestipol (10 g twice daily), para-aminosalicylic acid-C (6-8 g day in 3 divided doses), and oxandrolone (2.5 mg 3 times daily), was taken for a 3-month period, followed by 2 months without hypolipidemic drug therapy (control) before the next agent was begun. Subjects were randomly allocated to one of 3 drug sequence groups according to an experimental design that balanced the order in which clofibrate, colestipol, and PAS-C were dispensed among the 3 groups. Oxandrolone was the final drug given in every case because of concern over an anticipated longer duration of effect, which, in fact, was unfounded.

Laboratory Procedures

All blood samples were drawn from an antecubital vein, after an overnight (12-14 hr) fast, into Vacutainer (R) tubes containing disodium EDTA, 1.5 mg/dl, according to Lipid Research Clinics protocols (7). Cells were removed by low-speed centrifugation at 4 C within 2 hr, and the plasma was stored at 4 C and processed within 72 hr for lipid and lipoprotein lipid analysis.

Cholesterol and triglyceride were measured by AutoAnalyzer II techniques (7) in whole plasma and plasma fractions. For cholesterol analysis, the coefficient of variation was less than 2% and accuracy within 2% of the target values; for triglyceride analysis, the coefficient of variation was less than 4% and accuracy was within 3%.

The lipoprotein fractions were obtained using a combination of ultracentrifugation and chemical precipitation techniques (7). Plasma without density adjustment was subjected to centrifugation at $105{,}000 \times g$ for 18 hr at 10 C. The $d<1.006$ fraction (VLDL) was separated by a tube-slicing technique and this portion and the $d>1.006$ fraction, containing LDL and HDL, were adjusted to the original plasma volume with 0.15 M saline before lipid analysis.

Another aliquot of plasma was treated with heparin and manganese according to standard Lipid Research Clinics procedure with manganese at 46 mM final concentration (7). The precipitated apoB-containing lipoproteins, primarily VLDL and LDL, were sedimented by centrifugation; cholesterol and triglyceride values were measured in the supernatant solution.

Cholesterol and triglyceride values in the

HDL fraction were corrected for the dilution incurred as a result of adding the precipitating reagents. LDL cholesterol and triglyceride were calculated as the difference between their respective values in the d> 1.006 fraction (LDL plus HDL) and the HDL fraction. VLDL triglyceride and cholesterol values reported were calculated indirectly as the difference between their values in the total plasma and the d> 1.006 plasma fraction.

Statistical Analysis

Differences in lipids, lipoproteins and adherence among drug treatments were statistically evaluated by the analysis of variance techniques. A 2-factor design for repeated measures (8) was used to test for drug treatment and sequence effects and for any interaction between the drugs and their order of administration. Because of unequal sample sizes in the 4 treatment groups, a least-squares solution was used to estimate treatment effects. Significant mean differences between each drug and its control were determined using multiple comparison techniques. Measurements for triglyceride in whole plasma and the lipoprotein fractions and VLDL cholesterol were transferred to the logarithmic scale for statistical testing. Cochran's Q test (9) for correlated proportions was used with adjustment for multiple testing to determine which side effects were significantly associated with each drug. A detailed description of the statistical analyses used in these studies appeared as supplementary material in the earlier report (1).

RESULTS

Individual and summary data for plasma and lipoprotein cholesterol and triglyceride concentrations by treatment are given in Tables 1-2, respectively. A statistical comparison among the 3 baseline visits and each inter-drug control period disclosed no significant differences in the whole plasma and lipoportein lipid levels among the off-drug periods. The statistical tests applied to the data in Table 1 compared the mean of the 2 visits immediately preceding each drug treatment with the respective mean of the 3 visits on each drug. Specific analyses also failed to disclose any significant drug sequence effects or carry-over effects of any given drug on the response to the next drug in the sequence.

Clofibrate

Treatment with clofibrate at 2 g/d was associated with a significant increase in reports of nausea (43%). Nevertheless adherence was high (91 ≠ 9%, mean = S.D.). Significant decreases were recorded in mean plasma cholesterol (-16%) and triglyceride (-15%), VLDL-cholesterol (-61%) and VLDL-triglyceride (-61%), while HDL-cholesterol increased (+20%). The mean LDL cholesterol/HDL cholesterol ratio decreased (-24%). In only 3 subjects (one each with FHTG, BBD, and UNCL) was the fall in VLDL-cholesterol accompanied by a rise in LDL-cholesterol. In the subject with FHTG (who had the lowest baseline LDL cholesterol), this rise was dramatic (203%). If this exceptional result is excluded, the reduction in LDL-cholesterol with clofibrate averaged 13%. All but 3 subjects (two FH and one UNCL) experienced an increase in HDL cholesterol.

Colestripol

Treatment with colestipol at 20 g/d was associated with a significant increase in constipation (58%) and nausea (58%). Nevertheless, adherence averaged 89% ± 12% of the prescribed dose. Significant decreases were recorded in mean plasma cholesterol (-15%) and LDL-cholesterol (-22%), while VLDL-triglyceride increased (+41%). The mean LDL-cholesterol/HDL-cholesterol ratio decreased (-25%). Two individuals (one BBD and one UNCL) showed a rise in plasma cholesterol, while 3 subjects (one each with FH, FCHL, and UNCL) had lower triglyceride levels on colestipol.

PAS-C

Treatment with PAS-C at 6-8 g/d was associated with significantly increased reports of diarrhea and, in spite of dosage adjustments to alleviate this complaint, adherance appeared most erratic on this agent (81 ≠ 17%, based on 8 g/d). During PAS-C treatment significant decreases were recorded in VLDL-cholesterol (-30%), and VLDL-triglyceride (-29%). The LDL-cholesterol/HDL-cholesterol ratio did not change significantly. The responses to PAS-C were the least consistent in the study: two UNCL subjects had higher plasma cholesterol values while on PAS-C; these two, another UNCL, two BBD, and one FCHL had higher LDL-cholesterol levels during treatment with PAS-C.

Oxandrolone

Treatment with oxandrolone at 7.5 mg/d produced no significant side effects, and adherence was high (92 ≠ 13%). Oxandrolone significantly affected all lipid parameters. Mean plasma cholesterol increased 7%, and LDL-cholesterol increased 45% (25% excluding the subject with FHTG, who experienced a 298% rise), as did LDL-triglyceride (+24%). De-

TABLE 1

Individual Plasma and Lipoprotein Lipid Data Before Drug Represents Mean of Two Visits: During Drug Represents Mean of Three Vists
(C = Cholesterol; TG = Triglyceride, all in mg/dl)

Subject No.	Age	Sex	ASCVD[a]	Genetic[b] Classification	Before drug								During clofibrate							
					Plasma		VLDL		LDL		HDL		Plasma		VLDL		LDL		HDL	
					C	TG	C	TG	C	TG	C	TG	C	TG	C	TG	C	TG	C	TG
1	27	F	0	FH	361	113	51	69	266	39	44	6	294	84	23	41	224	41	47	2
2	29	M	0	FH	361	147	25	100	300	37	37	10	313	89	9	44	254	34	50	10
3	42	F	0	FH	413	98	14	44	306	34	92	20	344	69	10	23	261	35	73	11
4	41	F	0	FH	342	79	21	37	285	31	36	9	298	61	8	25	248	30	41	11
5	55	F	0	FH	353	126	13	58	262	48	78	19	231	88	11	38	157	31	70	19
6	36	M	0	BBD	198	163	53	127	94	26	48	10	231	207	58	159	120	36	53	12
7	48	M	+	BBD	358	652	221	602	117	36	20	14	224	244	78	205	109	31	37	8
8	50	M	0	BBD	227	200	67	152	108	35	48	11	159	104	28	61	79	32	53	11
9	66	F	0	FCHL	298	308	68	232	185	57	45	19	229	138	21	64	147	56	61	19
10	55	M	+	FHTG	200	628	119	563	55	35	24	30	219	140	19	73	167	53	33	14
11	35	M	0	UNCL	244	137	25	100	186	30	33	6	213	111	20	72	161	32	33	7
12	35	M	0	UNCL	288	156	32	113	216	34	39	10	266	138	19	85	208	40	37	13
13	48	F	0	UNCL	236	153	28	92	163	45	42	17	256	132	16	66	187	48	52	18
14	36	M	0	UNCL	346	411	102	333	213	63	31	15	311	129	15	78	252	43	44	9

Subject No.	Age	Sex	ASCVD[a]	Genetic[b] Classification	During colestipol								During PAS-C							
					Plasma		VLDL		LDL		HDL		Plasma		VLDL		LDL		HDL	
					C	TG	C	TG	C	TG	C	TG	C	TG	C	TG	C	TG	C	TG
1	27	F	0	FH	275	150	32	100	201	40	42	10	–	–	–	–	–	–	–	–
2	29	M	0	FH	334	161	28	110	262	39	44	11	305	100	10	62	258	28	42	9
3	42	F	0	FH	401	99	13	36	296	47	91	16	400	79	6	29	307	33	88	16
4	41	F	0	FH	291	119	16	83	235	30	40	7	288	54	10	19	247	29	32	6
5	55	F	0	FH	299	132	16	68	200	41	77	23	305	105	11	42	218	41	75	22
6	36	M	0	BBD	202	159	53	126	94	24	56	9	213	182	74	143	95	26	50	13
7	48	M	+	BBD	–	–	–	–	–	–	–	–	305	816	240	750	88	44	23	23
8	50	M	0	BBD	195	181	59	146	83	31	53	4	167	122	48	87	71	25	48	10
9	66	F	0	FCHL	241	288	52	223	144	44	45	21	263	208	31	124	168	59	64	25
10	55	M	+	FHTG	–	–	–	–	–	–	–	–	161	309	67	313	71	51	23	26
11	35	M	0	UNCL	193	155	27	121	130	24	36	11	256	136	25	98	196	29	35	9
12	35	M	0	UNCL	202	138	23	96	143	31	36	11	285	133	26	88	227	35	32	10
13	48	F	0	UNCL	–	–	–	–	–	–	–	–	244	166	21	98	184	55	39	13
14	36	M	0	UNCL	311	629	166	553	112	50	33	26	302	214	41	147	228	52	33	16

[a]ASCVD: Arteriosclerotic Cardiovascular Disease.
[b]FH: Familial Hypercholesterolema. BBD: Broad-β Disease. FCHL: Familial Combined Hyperlipidemia. FHTG: Familial Hypertriglyceridemia. UNCL: Unclassified.

TABLE 1 (Continued)

Subject No.	Age	Sex	ASCVD[a]	Genetic[b] Classification	During oxandrolone							
					Plasma		VLDL		LDL		HDL	
					C	TG	C	TG	C	TG	C	TG
1	27	F	0	FH	379	132	24	73	329	52	26	7
2	29	M	0	FH	396	108	26	50	316	55	24	3
3	42	F	0	FH	444	66	6	17	701	40	38	9
4	41	F	0	FH	332	54	7	15	298	35	26	4
5	55	F	0	FH	–	–	–	–	–	–	–	–
6	36	M	0	BBD	–	–	–	–	–	–	–	–
7	48	M	+	BBD	375	793	232	725	124	56	19	12
8	50	M	0	BBD	154	112	24	68	84	34	45	10
9	66	F	0	FCHL	–	–	–	–	–	–	–	–
10	55	M	+	FHTG	276	167	34	108	219	55	23	4
11	35	M	0	UNCL	333	125	18	65	299	56	16	4
12	35	M	0	UNCL	328	129	17	67	297	57	14	4
13	48	F	0	UNCL	317	134	16	52	278	66	23	16
14	36	M	0	UNCL	395	219	55	154	308	54	33	11

[a]ASCVD: Arteriosclerotic Cardiovascular Disease.
[b]FH: Familial Hypercholesterolema. BBD: Broad-β Disease. FCHL: Familial Combined Hyperlipidemia. FHTG: Familial Hypertriglyceridemia. UNCL: Unclassified.

TABLE 2

Effect of Clofibrate, Colestipol, PAS-C and Oxandrolone on Plasma and Lipoprotein Lipids (all in mg/dl)

No, of Subjects		Baseline	Treatment							
			Clofibrate		Colestipol		PAS-C		Oxandrolone	
			before	during	before	during	before	during	before	during
		14	14		12		14		11	
Plasma Cholesterol (C)	Mean	302	307	257[b]	316	268[b]	302	285	314	336[a]
	SD	70	69	50	75	62	77	85	81	78
	Median	320	324	246	310	283	320	286	332	330
C-VLDL	Mean	58	61	24[c]	35	44	66	46[a]	57	42[a]
	SD	57	55	20	20	41	80	63	98	64
	Median	41	47	19	27	30	31	28	28	64
C-LDL	Mean	199	203	178[b]	205	160[a]	195	188	208	246[b]
	SD	82	76	62	88	75	90	79	86	99
	Median	200	213	164	220	143	187	209	219	284
C-HDL	Mean	44	41	49[b]	47	50	45	44	47	26[c]
	DS	18	17	12	17	18	16	19	17	9
	Median	41	36	48	45	43	43	37	43	24
Plasma Triglyceride (T)	Mean	237	254	124[c]	206	200	255	209	268	185[a]
	SD	192	184	51	199	144	409	172	356	206
	Median	151	151	120	155	156	146	134	141	129
T-VLDL	Mean	183	189	74[c]	104	147[a]	201	143[a]	218	127[c]
	SD	188	173	50	60	136	224	172	352	202
	Median	107	108	69	100	115	112	93	93	67
T-LDL	Mean	39	42	39	40	37	41	40	41	51[b]
	SD	10	16	8	10	9	15	17	13	12
	Median	35	35	35	41	38	35	35	37	55
T-HDL	Mean	14	14	12	15	16	14	14	16	9[b]
	SD	6	7	5	7	10	7	6	9	4
	Median	12	13	11	15	13	12	13	12	9
C-LDL/C-HDL	Mean	4.84	5.11	3.88[a]	4.68	3.54[a]	4.55	4.67	4.77	9.96[c]
	SD	2.04	2.03	1.59	1.91	1.42	1.96	2.18	2.10	5.82
	Median	4.91	5.59	4.18	4.10	3.32	4.31	4.55	4.51	10.38

[a]$p<.05$.
[b]$p<.01$.
[c]$p<.005$.

creases occurred in mean plasma triglyceride (-31%), VLDL-cholesterol (-26%), VLDL-triglyceride (-42%), HDL-cholesterol (-45%) and HDL-triglyceride (-43%). As a reflection of the contrasting changes in LDL and HDL, the mean LDL-cholesterol/HDL-cholesterol ratio increased by 109%. HDL-cholesterol declined in all subjects, even those with depressed levels at baseline. The expected (10) inverse correlation between HDL-cholesterol and total or VLDL-triglyceride exhibited at baseline (r_s ca. 0.45 [Spearman's rank correlation coefficient]) and maintained during each of the other 3 drug treatments was not observed during oxandrolone therapy, (r_s = .006), while a negative relationship between HDL-cholesterol and LDL-triglyceride emerged (r_s = -0.87). Three subjects (two with FH and one UNCL) had higher VLDL-cholesterol during oxandrolone. One UNCL subject had higher plasma and HDL-triglyceride values during oxandrolone, LDL-triglyceride did not increase in 4 subjects (2 UNCL and 1 each with FH and FHTG), and VLDL-triglyceride did not decline in 2 participants (1 with FH and other UNCL).

Inconsistencies in Lipid Responses

Given the heterogeneity of the subjects enrolled in this study, the frequency of deviation from the average response of the group to the 4 drugs was small, and no pattern of deviation clearly correlated with the genetic classification of the participants. The average number of inconsistencies in each subject relative to the group response was 2-3 for the 8 plasma and lipoprotein lipids during the 4 drug treatments (out of 32 possible deviations). One UNCL subject had 10 such inconsistencies and one with BBD had 5. When the responses of the 5 with FH were specifically compared with the remainder of the group, qualitative similarities were almost uniformly seen (exceptions being attributable to the aberrant responses of the subject with FHTG already noted).

DISCUSSION

A detailed review of the lipid and lipoprotein effects and side effects of each of the 4 agents under investigation in this study is beyond the scope of this report. These effects have been summarized elsewhere (1,10-12) and a previous description of the effects of these drugs on HDL, its principal aplipoproteins, and total plasma cholesterol and triglyceride in this study has been published (1). Certain aspects of the present study that distinguish it from those previously described, however, are noteworthy.

First, no previous study has allowed direct comparison, within the same group of dyslipoproteinemic subjects, of these 4 (or any 4) agents. Hence, previous quantitative estimates of efficacy of these 4 drugs have required extrapolation from single drug or two-drug studies.

Second, the drugs chosen for this study were selected for their known diversity to permit inferences to be drawn as to their mechanisms of action in this carefully characterized, heterogeneous group of subjects. In this regard the outcome was not fully anticipated: in spite of the heterogeneity of the subjects, whether classified genetically or according to lipoprotein distribution, the results for each drug were generally similar among the participants regardless of classification. Thus, though the study was designed to test a pharmacogenetic hypothesis (i.e., that the response to each drug would proceed according to the genetic classification of the subjects), this hypothesis was clearly not supported by the data. With the possible exception of the subset with FH, the sample size of no subgroup permitted a definitive rejection of this hypothesis. Nevertheless, current knowledge would suggest that such an hypothesis is likely to prove overly simplistic, since with the advance of knowledge, the genetics of hyperlipidemia are proving exceedingly complex. For instance, at least 3 genetically distinct forms of homozygous FH have been defined at the subcellular level (13-15). Furthermore, and directly relevant to this hypothesis, certain forms of dyslipoproteinemia have been demonstrated to reflect combinations of genetic defects or polymorphisms; e.g. BBD appears to proceed from the combination of the homozygous isoapolipoprotein E_2 phenotype (16-18) with a cause for hyperlipidemia, usually FCHL (16,19) but occasionally FH (20).

Third, the general similarity of response between certain drug pairs was of interest. Clofibrate, for example, a drug traditionally prescribed for hypertriglyceridemia, was as efficacious in reducing cholesterol levels, even in those with FH, as was colestipol, though the latter agent was associated with a greater reduction in LDL cholesterol. The agents produced an equivalent amelioration of theoretical atherogenic risk, as reflected in ca. 25% decreases in the mean LDL-cholesterol/HDL-cholesterol ratio. Clofibrate was associated with a reduction in LDL cholesterol, even among most of the subjects with hypertriglyceridemia, contrary to a previous report (21). The reason for these differing results is unclear. However, given the prevalence of FCHL (22,23) and its associa-

tion with premature coronary disease (3), this discrepancy should be resolved through further studies, since definition of the drug or drugs of choice for this disorder is a matter of substantial importance. Such studies should also compare clofibrate with its analogues, e.g., gemfibrozil, bezafibrate, fenofibrate and others that are under intense eveluation at present, especially since the publication of the results of the three-country WHO-sponsored study that reported an unexpected increase in the total death rate with clofibrate (24).

Finally, this study clearly demonstrates that the treatment of hyperlipidemia with oxandrolone will adversely affect the already high theoretical atherogenic risk of nearly all subjects substantially. While triglyceride levels will decline (of questionable relationship to atherogenic risk in current thinking [25]), not only will LDL cholesterol levels rise but HDL cholesterol concentrations will be decreased by nearly half, even as the triglyceride falls. Hence, prudence in clinical judgment would suggest that oxandrolone be limited to the treatment of severe hypertriglyceridemia of a magnitude that places the patient at risk to develop pancreatitis, but which does not respond to alternative forms of hypotriglyceridemic therapy.

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REFERENCES

1. Cheung, M.C., Albers, J.J., Wahl, P.W., and Hazzard, W.R. (1980) Atherosclerosis 35, 215-228.
2. Gordon, T., Castelli, W.P., Hjortland, M.C., Kannel, W.B., and Dawber, T.R. (1977) Am. J. Med. 62, 707-714.
3. Brunzell, J.D., Schrott, H.G., Motulsky, A.G., and Bierman, E.L. (1976) Metabolism 25, 313-320.
4. Noble, R.P. (1968) J. Lipid Res. 9, 693-700.
5. Fredrickson, D.S., Morganroth, J., and Levy, R.I. (1975) Ann. Int. Med. 82, 150-157.
6. Warnick, G.R., Mayfield, C., Albers J.J., and Hazzard, W.R. (1979) Clin. Chem. 25, 279-284.
7. Lipid Research Clinics Program Manual of Laboratory Operations, (1974) Vol. 1, Lipid and Lipoprotein Analysis, Washington, D.C., US *Government* Printing Office, DHEW Pub No 75-628.
8. Winer, B.J. (1962) in Statistical Principles in Experimental Design, McGraw-Hill Book Company, New York, Chapter 7.
9. Marascuilo, L.A., and McSweeney, M. (1977) in Nonparametric and Distribution-free Methods for the Social Sciences, pp. 176, Brooks/Cole Publishing Co.
10. Grundy, S.M. (1982) Med. Clin. N. Am. 66, 519-535.
11. Kane, J.P., and Malloy, M.J. (1982) Med. Clin. N. Am. 66, 537-550.
12. Albers, J.J., Cheung, M.C., and Hazzard, W.R. (1978) Metab. Clin. Exp. 27, 479.
13. Breslow, J.L., Spaulding, D.R., Lux, S.E., Levy, R.I., and Less, R.S. (1975) New Eng. J. Med. 293, 900-903.
14. Goldstein, J.L., Dana, S.E., Brunschede, G.Y., and Brown, M.S. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 1092-1096.
15. Brown, M.S., Kovanen, P.T., and Goldstein, J.L. (1981) Science 212, 628-635.
16. Utermann, G., Vogelberg, K.H., Steinmetz, A., Schoenborn, W., Pruin, N., Jaeschke, M., Hees, M., and Canzler, H. (1979) Clin. Genet. 15, 37-62.
17. Zannis, V.I., Just, P.W., and Breslow, J.L. (1981) Am.J. Hum. Genet. 3, 11-24.
18. Havel, R.J. (1982) Med. Clin. N. Am. 66, 441-454.
19. Hazzard, W.R., Warnick, G.R., Utermann, G., and Albers, J.J. (1981) Metabolism 30, 79-88.
20. Hazzard, W.R., Albers, J.J., Baron, P., Miller, N., Warnick, G.R., and Lewis, B. (1981) Lancet 1, 298-301.
21. Wilson, D.E., and Lees, R.S. (1972) J. Clin. Invest. 51, 1051-1057.
22. Goldstein, J.L., Hazzard, W.R., Schrott, H.G., Bierman, E.L., and Motulsky, A.G. (1973) J. Clin. Invest. 52, 1544-1568.
23. Boman, H., Hazzard, W.R., Albers, J.J., Cooper, M.N., and Motulsky, A.G. (1975) Am. J. Human Genet. 27, 19A.
24. Oliver, M.R., Heady, J.A., Morris, J.N., and Cooper, J. (1978) Br. Heart J. 40, 1069-1179.
25. Lippel, K., Tyroler, H., Eder, H., Gotto, Jr., A., and Vahouny, G. (1981) Arteriosclerosis 1, 406-417.
26. Brunzell, J.D., and Bierman, E.L. (1982) Med. Clin, N. Am. 66, 455-468.

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Increased Lysolecithin Acyltransferase Activity in the Plasma of Type II Hyperlipoproteinemic Patients

PAPASANI V. SUBBAIAH[a,*], and JAMES T. OGILVIE[b], [a]*Department of Medicine, University of Washington, Providence Medical Center, 500 17th Avenue, Seattle, WA 98124 and* [b]*Northwest Lipid Research Clinic, Department of Medicine, University of Washington, Seattle, WA 98104*

ABSTRACT

Lecithin-cholesterol acyltransferase (LCAT) in human plasma has been shown to acylate lysolecithin to lecithin in presence of low density lipoprotein (LDL). To determine the physiological importance of LDL in activating lysolecithin acyltransferase (LAT) activity, we assayed the LAT activity in 18 hypercholesterolemic (Type II) patients and 15 control subjects. The enzyme activity was about 60% higher in the patients compared with the controls. On the other hand, the LCAT activity, measured by 2 different procedures, as well as enzyme mass, determined by radioimmuno assay, were comparable in the controls and hypercholesterolemics. The LAT activity was highly correlated with LDL levels in the plasma, but the LCAT activity and the enzyme mass had no correlation with the LDL levels. These results show that the plasma LDL is the rate-limiting activator of the enzyme, and pathological conditions, resulting in higher LDL levels, also cause higher LAT activities.
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INTRODUCTION

The importance of LDL as the principal carrier of plasma cholesteryl esters, and as an indicator of risk of atherosclerosis (1,2), is well known. In addition to carrying cholesterol to peripheral tissues, LDL has been shown to have a significant effect on the morphology of the erythrocytes, presumably by activating the membrane-associated phosphatase (3), and also inhibits the lymphocyte activation induced by phytohemagglutenin (4). We have shown recently that LDL is a specific activator of the LAT reaction carried out by LCAT (EC 2.3.1. 43) (5,6). This enzyme, which is responsible for the production of most of the cholesteryl esters and lysolecithin in the plasma, can acylate lysolecithin back to lecithin, but only in the presence of LDL (6,7). That this requirement of LDL for lysolecithin esterification is a physiological one is shown by the fact that in abetalipoproteinemic patients, the LAT activity is only about 5-6% of the control subjects whereas the LCAT activity is the value in about 40% of the control levels (8). Moreover, the addition of normal LDL but not VLDL to abetalipoproteinemic plasma activates the LAT reaction up to 20-fold, while LCAT activity is stimulated about 4-fold, the same stimulation as with VLDL. These results showed that LDL is a specific activator of the LAT reaction. In this paper we provide evidence that when LDL is present in higher than normal amounts, as in Type II hyperlipemic patients, the LAT activity also increases. The enzyme activity correlated positively with the LDL levels of the plasma, whereas the enzyme mass and cholesterol esterifying rate were not different from the control population. These results provide further proof that LDL is a physiological activator of the reaction and that the reaction is abnormal in patients with high levels of LDL.

*To Whom correspondence should be addressed.

MATERIALS AND EXPERIMENTAL METHODS

Patients

We analyzed plasma (drawn after a 12 hr, overnight fast) from 18 patients with Type II hyperlipidemia (4 females, 14 males) and 15 control subjects (4 females, 11 males). Two of the patients were of the mild Type IIb phenotype, the remaining 16 were Type IIa. At the time of sampling for LAT analysis, 11 of the patients were taking lipid lowering medications, and 7 were on a controlled diet only. Medications used were: colestipol or cholestyramine 3, niacin 3, combined resin and niacin 3, and clofibrate 2. Before medication, each patient had demonstrated LDL cholesterol levels above the 95th percentile, adjusted for age and sex. Twelve of the patients' family histories demonstrated first-degree relatives with elevated lipids, premature cardiovascular disease or both. Four of the patients had tendonous xanthoma, and 4 showed evidence of cardiovascular disease. None of the patients showed abnormalities indicating hepatic, renal or thyroid disease, or diabetes. No significant differences in enzyme activites

were found between patients who were treated with drugs and those who were not treated. Informed consent was obtained from each subject, and the study was approved by the human subjects review committees of Providence Medical Center and the University of Washington. Blood samples were drawn in EDTA (7 mg/ml). The plasma was separated at 4 C and kept frozen at -20 C until the enzyme assays were performed. All the enzyme assays were done within 3 weeks of drawing blood.

LAT Assay

The enzymatic acylation of labeled lysolecithin by the plasma was measured essentially as described earlier (8) except that ^{3}H-glycerol-labeled lysolecithin (6) was used as substrate. The substrate (0.15 μmol/reaction) was first dispersed in 10 mM Tris-0.15 M NaCl-1 mM EDTA, pH 7.4, by vortexing. To each tube, 0.1 ml of the ^{3}H-lysolecithin dispersion, 20 μl of 0.1 M mercaptoethanol, and 0.13 ml of 1 mM EDTA containing 0.15 M NaCl, pH 7.4, were added, in that order. The solution was mixed and the reaction was started by adding 0.15 ml of plasma. Each plasma sample was assayed in duplicate, and control reactions containing no plasma were run simultaneously. The samples were incubated at 37 C for 60 min and the reaction was stopped by the addition of 1 ml of methanol. The lipids were extracted by the procedure of Bligh and Dyer (9). Lysolecithin and lecithin were separated on TLC plates using the solvent system chloroform/methanol/water (65:25:4 by vol). Lipids were visualized by a brief exposure to iodine vapors and the spots of lysolecithin and lecithin were scraped into scintillation vials. The radioactivity was determined in a Beckman LS-7000 liquid scintillation system after adding 0.5 ml water and 5.0 ml Aquasol (New England Nuclear, Boston, MA). The radioactivity in the samples was corrected for quenching using the H number (Beckman Instruments, Pato Alto, CA). The percentage of lysolecithin converted to lecithin was calculated, and the enzyme activities were expressed as nmoles of lysolecithin acylated per hour/ml of plasma. The molar esterification rate of lysolecithin was calculated by extrapolating the concentration of lysolecithin present in the reaction mixture (0.15 ml plasma) to 1 ml of plasma and multiplying by the percentage esterification rate. This method gives higher values of LAT activity than reported earlier (8), but does not affect the relative activities present in various subjects.

LCAT Assays

The assay for LCAT was performed essentially as described by Stokke and Norum (10), as modified by Lacko et al. (11), with the labeled cholesterol equilibrated with endogenous cholesterol as the substrate. The DTNB concentration present in the incubation with labeled cholesterol was 1.33 mM. The values obtained here are lower than those reported by Stokke and Norum (10) because of higher blank values in our experiments. Increasing the DTNB concentration resulted in the incomplete reversal of the inhibition by mercaptoethanol, thus decreasing the enzyme activities. Since we assayed samples from both controls and patints at the same time, these experimental problems should not impair the interperetation of the results. The lipid extractions and radioactivity determination were done as described by Bories et al. (12). LCAT activity was also assayed by the proteoliposome method of Chen and Albers (13), which is independent of the lipoprotein activators and correlates highly with LCAT mass as measured by radio immunoassay (14). Each reaction mixture contained 250 nmoles of lecithin, 12.6 nmoles of labeled cholesterol, 0.8 nmoles of apo A-I, 0.5% HSA, 5 mM mercaptoethanol, 6.25 mM Tris-Cl pH 7.4, and 20 μl of the plasma in a final volume of 400 μl.

Estimation of Lipids

Total plasma triglycerides were assayed by the procedure of Van Handel and Zilversmit (15). Total cholesterol and unesterified cholesterol were measured by the procedure of Zak et al. (16), after extraction and digitonin precipitation, as described by Parekh et al. (17). LDL cholesterol was estimated by derived β-quantification (Lipid Research Clinic manual), using the procedure of Friedwald et al. (18). Lysolecithin and lecithin were determined by phosphorus estimation (19) after separation on TLC plates using the solvent system chloroform/methanol/water (65:25:4). The estimation of LCAT mass was performed by Dr. John J. Albers by radioimmunoassay (14). Standard statistical methods were used to determine the significance of the differences and of the correlation coefficients (20).

RESULTS AND DISCUSSION

The lipid values of the plasma from the hypercholesterolemic and control subjects are shown in Table 1. As expected, the patients had higher LDL cholesterol levels and lower HDL levels. Their lecithin values were higher than the controls, but the lysolecithin or TG levels were not significantly different from the controls.

The LAT and the LCAT rates are shown in Table 2. The acylation of lysolecithin was signi-

TABLE 1

Lipid Levels of Hypercholesterolemic (Type II) and Normocholesterolemic Subjects[a]

Subjects	Age (range)	Whole plasma Cholesterol Free[b]	Whole plasma Cholesterol Total	HDL chol.	LDL chol.	Whole plasma TG	Whole plasma lysolecithin	Whole plasma licithin
Hypercholesterolemic (Type II) n = 18	39.11± 15.51 (10-62)	1.435± 0.544	7.438± 2.007	1.080± 0.380	5.786± 2.002	1.233± 0.601	0.164± 0.047	1.843± 0.396
Normocholesterolemic n = 15	28.67± 6.01 (16-37)	1.029± 0.256	4.378± 0.891	1.436± 0.252	2.178± 0.647	1.031± 0.521	0.170± 0.045	1.510± 0.284
P value	<0.05	<0.025	<0.001	<0.01	<0.001	>0.05	>0.05	<0.01

[a]All lipid values are expressed as μmoles/ml plasma ± S.D.
[b]Unesterified cholesterol.

TABLE 2

LCAT and LAT Activities and Enzyme Mass in Hypercholesterolemic (Type II) and Normal Subjects

Subjects	LCAT (Stokke & Norum) Assay	LCAT[a] (Proteoliposome) Assay	LAT	Enzyme mass[b] μg/ml plasma
	n moles of substrate acylated/hr/ml plasma			
Hypercholesterolemic (Type II) n = 18	43.76± 17.90	55.19± 8.97	21.39± 6.37	5.77± 1.32
Normal (Controls) n = 15	46.27± 14.02	63.37± 12.20	13.42± 3.48	5.47± 0.89
P	N.S.	N.S.	<0.001	N.S.

All values presented are mean ± S.D.
[a]Assays performed in 15 hypercholesterolemic patients and 11 normal subjects.
[b]Mass determined in 16 patients and 9 control subjects.

ficantly higher in the hyperlipoproteinemic patients than in the controls. The LCAT activity, however, was not different from controls, whether assayed by the esterification of labeled cholesterol preequilibrated with endogenous cholesterol (10) or by the proteoliposome assay, which is not affected by the plasma cholesterol concentration (13). Enzyme mass, as determined by the radioimmunoassay, was the same for the patients and the controls. As reported by other workers (21,22), our data also demonstrate that the LCAT activity of Type IIa patients is normal. The significantly higher activity of LAT is, therefore, not from increased levels of the enzyme.

As seen in Figure 1, the level of LAT activity correlates closely with LDL level in the plasma, both in control and Type IIa subjects. This correlation also exists for total cholesterol values of the plasma (Table 3). There was, however, a negative corelation in LAT activity and HDL cholesterol levels. The LAT activity also correlated significantly with plasma TG values. No correlation was found with plasma lysolecithin, LCAT activity, or LCAT mass. A significant correlation with lecithin values was found only when control and Type II subjects are combined.

In order to see if LDL cholesterol values correlated with LAT activity, independent of TG or lecithin levels of plasma, the partial correlation coefficients were calculated, keeping one of the variables constant. When holding the plasma TG levels constant, the LAT activity remains highly correlated with LDL cholesterol in all subjects grouped together ($r=0.814$; $p<0.01$), as well as in Type II subjects alone ($r=0.674$; $p<0.01$) or in controls alone ($r=0.660$; $p<0.01$). These results indicate that the relationships of LDL cholesterol and plasma TG on LAT activity are independent of each other. On the other hand, when calculating the partial

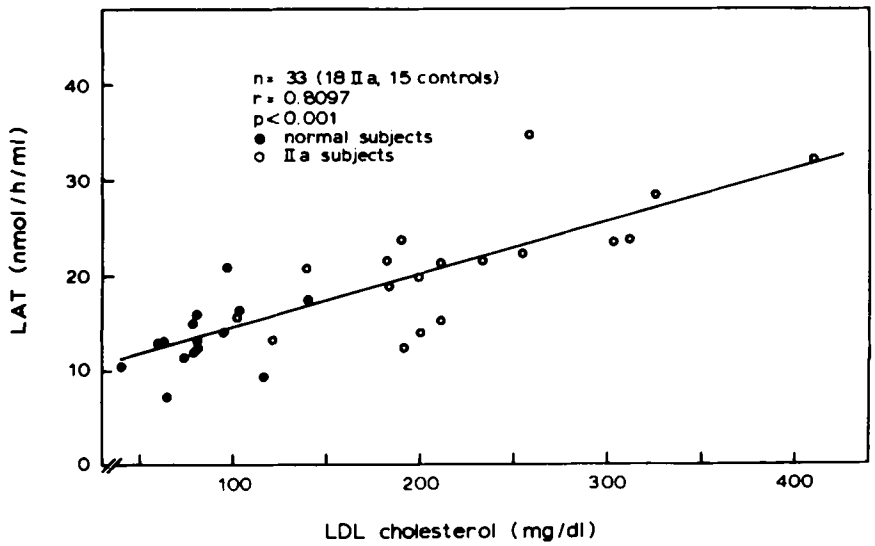

FIG. 1. Correlation of LAT activity with plasma LDL levels. The LAT activity was determined by the conversion of labeled lysolecithin to lecithin by the whole plasma as described in the text. LDL in each of the control plasma (•) and the hypercholesterolemic plasma (○) was determined by derived β-quantification (18). Two of the Type IIa subjects were of Type IIb (above 95th percentile TG) at the time of analysis.

TABLE 3

Correlation of LAT Activity with LCAT and Lipid Levels[a]

Variable	All subjects	Type II subjects	Normal subjects
	(n = 33)	(n = 18)	(n = 15)
LDL cholesterol	0.810***	0.721***	0.505*
HDL cholesterol	-0.547***	-0.354	-0.443
Whole plasma cholesterol	0.809***	0.735***	0.469
Whole plasma TG	0.605***	0.664**	0.616*
Whole plasma lysolecithin	0.216	0.407	0.185
Whole plasma lecithin	0.483**	0.357	0.150
LCAT activity	0.082	0.084	0.323
LCAT mass	0.150	0.035	0.347

[a]The coefficients of correlation were calculated according to the standard procedure (20). From these values, the t values were calculated according to the formula, $t = r\sqrt{(n-2)/(1-r^2)}$.

Significance levels: * $P < 0.025$. ** $P < 0.005$. *** $P < 0.001$. All other numbers have $P > 0.05$.

correlation coefficients of lecithin and LDL cholesterol with LAT, the correlation between LAT activity and lecithin observed in the total number of subjects (Table 3) did not persist if LDL cholesterol is kept constant (r=0.034). The correlation between LAT and LDL cholesterol, however, remains significant when the lecithin value is kept constant (r=0.743; $p<0.01$).

These results support the hypothesis that the LDL is a physiological activator of the LCAT reaction, and that the LDL levels in the plasma determine the rate of lysolecithin acylation. We had previously shown that, in patients with abetalipoproteinemia, the activity of LAT is very low and is stimulated up to 20-fold by the addition of normal LDL (8). The addition of LDL to normal plasma also stimulates the LAT activity of the plasma up to 100% (8), indicating that the LDL levels in normal plasma are not sufficient for maximal LAT activation. Thus, at higher LDL levels, as found in Type IIa hyperlipoproteinemia, the LAT activity is stimulated further without an increase in the LCAT mass or activity. The physiological importance of the LDL activation of the LAT reaction is also underscored by the observation that a small but significant amount of active

LCAT enzyme is associated with LDL in normal human plasma (23,24). The esterification of cholesterol can also take place on LDL surface (7,25). Whether the amount of LCAT associated with LDL fraction is increased in Type II hyperlipoproteinemia would be on interesting study. We do not know if the positive correlation found between the plasma TG levels and the LAT activity is from any effects of the TG on the enzyme activity, or is correlated with an unknown factor affecting both components.

The negative correlation between HDL cholesterol values and the LAT activities is not evident if the LDL cholesterol value is kept constant (partial correlation coefficient: -0.147). With HDL cholesterol constant, the LDL cholesterol value still correlates positively with LAT activity (r=0.721). These results suggest that the negative correlation between HDL and LAT is caused by the significant negative correlation between HDL cholesterol and LDL cholesterol (r=-0.590 for all subjects, -0.369 for Type II subjects and -0.616 for normal subjects), and not by a direct effect of HDL on LAT.

The results presented in this communication provide further evidence that LDL may play an important role in the metabolism of phospholipids in the plasma. Further studies are required to show whether different molecular species of lecithins are synthesized in hypercholeaterolemic individuals compared with the controls, and if the enzyme activity in the patients is reduced after hyperlipemia is treated with drugs or diet.

ACKNOWLEDGMENTS

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REFERENCES

1. Lewis, B. (1980) in Diet and Drugs in Atherosclerosis (Noseda, G., Lewis, B. and Paoletti, R., eds.) pp. 1-8, Raven Press, New York.
2. Gotto, A.M., Jr. (1979) in Atherosclerosis Reviews (Paoletti, R. and Gotto, A.M. Jr., eds.) Vol. 4, pp. 17-28, Raven Press, New York.
3. Hui, D.Y., and Harmony, J.A.K. (1979) Biochim. Biophys. Acta 550, 425-434.
4. Hui, D.Y., and Harmony, J.A.K. (1980) Proc. Natl. Acad. Sci. USA 77, 4764-4768.
5. Subbaiah, P.V., and Bagdade, J.D. (1979) Biochim. Biophys. Acta 573, 212-217.
6. Subbaiah, P.V., Albers, J.J., Chen, C.H., and Bagdade, J.D. (1980) J. Biol. Chem. 225, 9275-9280.
7. Subbaiah, P.V., Chen, C.H., Albers, J.J., and Bagdade, J.D. (1982) Atherosclerosis 45, 181-190.
8. Subbaiah, P.V. (1982) Metabolism 31, 294-298.
9. Bligh, E.G., and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911-917.
10. Stokke, K.T., and Norum, K.R. (1971) Scand. J. Clin. Lab. Invest. 27, 21-27.
11. Lacko, A.G., Rutenberg, H.L., and Soloff, L.A. (1973) Biochem. Med. 7, 178-183.
12. Bories, P.C., Subbaiah, P.V., and Bagdade, J.D. (1982) Nephron 32, 22-27.
13. Chen, C.H., and Albers, J.J. (1982) J. Lipid Res. 23, 680-691.
14. Albers, J.J., Adolphson, J.L., and Chen, C.H. (1981). J. Clin. Invest. 67, 141-148.
15. Van Handel, E., and Zilversmit, D.B. (1957) J. Lab. Clin. Med. 50, 152-157.
16. Zak, B., Dickenman, R.C., White E.G., Burnett, H., and Cherney, P.J. (1954) Am. J. Clin. Pathol. 24, 1307-1315.
17. Parekh, A.C., Caranto, M., Mathur, R.K., and Jung, D.H. (1976) Ann. Clin. Lab. Sci. 6, 423-429.
18. Friedwald, W.T., Levy, R.I., and Fredrickson, D.S. (1972) Clin. Chem. 18, 499-502.
19. Bartlett, G.R. (1959) J. Biol. Chem. 234, 466-468.
20. Snedecor, G.W., and Cochran, W.G. (1976) Statistical Methods, 6th ed. Iowa State University Press, Ames. I.A.
21. Wallentin, L. (1977) Atherosclerosis 26, 233-248.
22. Cicuianu, M., Opincaru, A., and Tapalaga, D. (1978) Clin. Chim. Acta 85, 73-79.
23. Chen, C.H., and Albers, J.J. (1982) Biochem. Biophys. Res. Commun. 107, 1091-1096.
24. Chung, J., Abano, D., Byrne, R., and Scanu A.M. (1982) Atherosclerosis 45, 33-41.
25. Barter, P.J. (1983) Biochim. Biophys. Acta 751, 261-270.

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Cholesterol Binding Capacity of Fiber From Tropical Fruits and Vegetables

ERIC D. LUND, *U.S. Citrus and Subtropical Products Laboratory,*[1] *P.O. Box 1909, Winter Haven, FL 33883*

ABSTRACT

The cholesterol binding capacity of 28 fiber samples from a variety of the more common tropical fruits and vegetables was determined. The binding capacity of cholestyramine, cellulose, lignin, guar gum and citrus pectin were also determined. Capacities were evaluated by an in vitro method that simulates the effect of the human digestive system on fiber using a series of enzymatic treatments before the binding was determined. Binding values varied from 3% for a soluble fraction of cassava to 84% for cholestyramine. Values for most fruit and vegetable fiber samples were less than or ca. equal to cellulose or lignin (20% and 16%, respectively). Apart from cholestyramine, sweet potato was the most effective binder (30%). Citrus pectin, at 8%, was a relatively poor binder. The capacity of guar gum (17%) was slightly less than cellulose. These data do not support the conclusion from in vivo studies that the hypocholesteremic effects observed for citrus pectin and guar gum are the result of the direct binding of cholesterol or bile acids in the large intestine.

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INTRODUCTION

Fiber from fruits, legumes and vegetables has been associated with reduced levels of plasma cholesterol (1,2). A tentative correlation between fiber and colorectal cancer has also been proposed (1,3). One of the primary mechanisms proposed for these effects is the direct binding of cholesterol, bile acids and related compounds in the large intestine.

The evaluation of the binding capacity of dietary fiber for cholesterol and bile acids is conveniently carried out by in vitro methods. Previous in vitro studies (4) have shown that substances such as alfalfa, wheat bran, lignin and cellulose are relatively ineffective. Cholestyramine (a commercial resin used for blood cholesterol reduction) is very effective. Guar gum (a high viscosity polyelectrolyte similar to pectin) is generally not as effective as cholestyramine, but better than lignin or cellulose.

These binding effects are correlated with changes in blood cholesterol shown in feeding studies (1). A recent feeding study showed that several tropical tubers (some of which contain unusual types of pectins and gums) reduced cholesterol levels substantially in the blood and in some of the internal organs of rats (5). Since many tropical fruits and vegetables contain pectins and gums in relatively high concentrations, the evaluation of the binding capacity of fiber from these foods may reveal improved sources of lipid-sequestering substances.

One of the complicating factors in previous in vitro studies has been the degradation of fiber in the digestive system (5). Enzymes produced by intestinal bacteria degrade pectin and hemicellulose (6) extensively. Even cellulose is partially hydrolyzed, leaving lignin substantially unchanged (7). In view of these problems, an unmodified fiber fraction may not show a realistic picture of the properties of the fiber residue as it exists in the large intestine.

In the present study, the direct binding of cholesterol by fiber fractions from a variety of tropical fruits and vegetables was evaluated. A modified in vitro procedure was employed that simulates the enzymatic degradation occurring as the fiber passes through the digestive system.

EXPERIMENTAL PROCEDURES

Materials

Yams, taro and tropical sweet potato were obtained from the Mayaguez Institute of Tropical Agriculture, Mayaguez, Puerto Rico. Carambolas, sapodillas and mangos were obtained from the USDA Subtropical Horticultural Research Station, Miami, FL. The remaining fruits and vegetables were purchased at local markets. Cholestyramine, cellulose, lignin, guar gum and citrus pectin were obtained from various commercial sources (see Table 1).

Enzymes

The 5 enzymes listed below were used in this study. The activities reported were those de-

[1]Southern Region, U.S. Department of Agriculture, Agricultural Research Service. Mention of a Trademark or proprietary product is for identification only and does not imply a guarantee or warranty of the product by the U.S. Department of Agriculture over other products that may also be suitable.

TABLE 1

Cholesterol Binding Capacity

Sample	AIR used mg	Enzymatic fiber in AIR (20 mg total)		Cholesterol recovered (1.70 mg added)		Binding capacity % of total in residue[a]	Total cellulose + lignin in AIR mg	Relative capacity per unit lignocellulose[b]
		Sol mg	Insol mg	Supernatant mg	Residue mg			
Cholestyramine[c]	20	–	20.0	0.27 ± 0.03	1.43 ± 0.14	84 ± 9	–	–
Sweet potato, flesh	131	8.2	11.8	0.65	0.51	30	6.7	4.5
Carambola (Tean Ma)	22	5.1	14.9	1.21	0.49	29	8.3	3.3
Mango (Keitt)	32	7.5	12.5	1.21	0.48	28	8.0	3.5
Sweet potato, flesh[d]	110	8.8	11.2	0.65	0.43	25	7.1	3.5
Sweet potato, peel	32	6.5	13.5	1.29	0.41	24	7.1	3.4
Sapodilla	22	3.7	16.3	1.34	0.41	23	18.7	1.2
Calabaza (Cuban pumpkin)	36	8.3	11.7	1.36	0.41	23	7.1	3.3
Taro (dasheen)	93	9.0	11.0	1.28	0.37	22	3.7	5.9
Sweet potato, flesh, enzymatic insoluble	20	–	20.0	1.31	0.37	22	–	–
Avocado[e]	28	7.7	12.3	1.37	0.36	21	8.2	2.6
Cassava (manioc, tapioca)	205	4.8	15.2	0.58	0.36	21	5.0	4.2
Mango (Tommy Atkins)	27	7.3	12.7	1.57	0.39	20	8.6	2.3
Cellulose[f]	20	–	20.0	1.36 ± 0.14	0.34 ± 0.03	20 + 2	20.0	1.0
Banana[g]	29	9.0	11.0	1.17	0.27	19	5.0	3.8
Dioscorea esculenta, var. Doli (yam)	167	4.2	15.8	0.39	0.31	18	4.7	3.8
Dioscorea esculenta, var. Muni[h] (yam)	312	3.0	17.0	1.28	0.31	18	9.7	1.8
Guar gum[i]	20	–	–	1.46	0.29	17	–	–
Grapefruit, sections enzymatic insoluble	20	–	20.0	1.26	0.27	16	–	–
Grapefruit (Marsh) sections[j]	27	12.8	7.2	1.31	0.27	16	4.5	3.6
albedo	20	9.4	10.6	1.43	0.27	16	5.3	3.0
Lignin[k]	20	–	20.0	1.41	0.27	16	20.0	0.8
Dioscorea alata, var. Forastero (yam)	176	4.8	15.2	0.99	0.26	15	5.6	2.7
Date	23	5.0	15.0	1.57	0.25	14	7.9	1.8
Cassava, enzymatic insoluble	20	–	20.0	1.51	0.22	13	–	–
Pineapple	27	3.3	16.7	1.36	0.20	12	8.7	1.4
Dioscorea esculenta, var. Beti (yam)	157	3.9	16.1	0.70	0.20	12	3.3	3.6
Guava[l]	20	4.7	15.3	1.50	0.20	12	5.2	2.3
Papaya	24	8.3	11.7	1.59	0.20	11	8.6	1.3
Dioscorea alata, var. Veeven (yam)	109	5.0	15.0	1.28	0.17	10	4.7	2.1
Kiwi	22	7.8	12.2	1.48	0.17	10	5.3	1.9

TABLE 1 (continued)

Sample	AIR used mg	Enzymatic fiber in AIR (20 mg total) Sol mg	Insol mg	Cholesterol recovered (1.70 mg added) Supernatant mg	Residue mg	Binding capacity % of total in residue[a]	Total cellulose + lignin in AIR mg	Relative capacity per unit lignocellulose[b]
Coconut[m]	26	4.7	15.3	1.62	0.17	9	9.8	0.9
Malanga[n]	113	5.7	14.3	1.00	0.15	9	3.0	3.0
Citrus pectin[o]	20	–	–	1.72	0.15	8	–	–
Dioscorea alata, var. Guunung (yam)	98	3.1	16.9	0.78	0.14	8	5.0	1.6
Sweet potato, flesh, enzymatic soluble	20	20.0	–	1.46	0.14	8	–	–
Dioscorea rotundata (yam)	70	12.7	7.3	1.36	0.12	7	1.02	6.9
Grapefruit, sections, enzymatic soluble	20	20.0	–	1.43	0.10	6	–	–
Cassava, enzymatic soluble	20	20.0	–	1.65	0.05	3	–	–

[a]Percentage of total cholesterol in centrifuge residue. Cholestyramine and cellulose were determined in triplicate. The percent variability for these values is shown in the table. The remaining values are based on a single determination (estimated percent variability ± 20%).

[b]Relative capacity as percentage of total cholesterol in centrifuge residue divided by the total value for cellulose and lignin in the AIR.

[c]Dowex 1x2-400 ion exchange resin (Dowex-1-chloride strongly basic anion exchange resin, 2% cross linking, 200-400 dry mesh). Washed with 1N HCl, 1N NaOH and water.

[d]Obtained from Puerto Rico.

[e]Grown in California.

[f]Solka-Floc (Brown Co., Berlin, NH. Extracted with ether).

[g]Enzymatic fractions: insoluble 1.40 ± 0.01, soluble 1.15 ± 0.07, dry wt. 23.21 ± 0.20, AIR 3.63 (% of fresh wt. ± SD).

[h]Combined enzymatic fractions analyzed (17 mg insoluble +3 mg soluble).

[i]Hercules, Type FG 30-70, low viscosity, food grade. (Hercules, Inc., Middletown, NY).

[j]Sections only, intersegmental mambrane not included.

[k]Indulin AT Kraft pine lignin polymer, Westvaco, Charleston Heights, SC.

[l]Enzymatic fractions: insoluble 5.89 ± 0.05, soluble 1.81 ± 0.09, dry wt. 14.24 ± 1.00, AIR 7.50 (% of fresh wt. ± SD).

[m]Grown in the Bahamas.

[n]Small corm.

[o]Pectin N.F. pure citrus pectin No. 3442, Sunkist Growers, Inc., Corona, CA.

scribed by the commercial supplier.

The glycoamylase (amyloglucosidase) was Sigma A-7255 with an activity of 5,000 to 10,000 units/gm (one unit liberates 1 mg of glucose from starch in 3 min at pH 4.5 and 55 C).

The pancreatin was Sigma P-1750 with an activity equivalent to 4X N.F. specifications (according to N.F. specifications, the enzyme must convert no less than 25 times its weight of potato starch into soluble carbohydrates in 5 min in water at 40 C and not less than 25 times its weight of casein into proteoses in 60 min).

The pepsin was Sigma P-7000 with an activity of 1,200 to 2,000 units per mg protein.

The hemicellulase was U.S. Biochemical No. 16870 with an activity of 2,500 units/gm (1 unit produces a change in the relative fluidity of 1 in a defined locust bean substrate).

The pectinase was U.S. Biochemical No. 19960 with an activity of ca. 600 APU per gm (1000 APU per ml gives a 50% reduction in the viscosity of an apple pectin solution in 60 min at 25 C and pH 3.8).

Fiber Composition

Composition data (cellulose, hemicelluose, lignin and enzymatic soluble and insoluble fraction) were taken from previous studies from this laboratory on the dietary fiber composition of tropical fruits and vegetables (8,9). These values were determined by the Van Soest detergent procedure and the Hellendoorn enzymatic analysis. Van Soest detergent values for banana and guava were taken from the literature (10). Enzymatic values for banana and guava were determined for this study by the Hellendoorn enzymatic analysis.

Preparation of Enzymatic Fractions

Banana and guava samples were digested with pepsin, glucoamylase and pancreatin, according to the Hellendoorn enzymatic procedure described in previous studies (8,9). The product was centrifuged and the supernatant mixed with a large excess of ethanol. The residue from the initial centrifugation was the insoluble fraction. The precipitate produced by the ethanol treatment was the soluble fraction.

Isolation and Enzymatic Pretreatment of Fiber Samples

Representative samples of fresh fruits and vegetables were blended and mixed with 800 g/4 l boiling 80% ethanol. After cooling, the alcohol insoluble residues (AIR) were isolated by filtration and dried at room temperature in an evacuated desiccator over $CaSO_4$. Final drying was carried out at 0.1 Torr (8,9).

The amount of each AIR sample used for the cholesterol binding assay was adjusted to contain 20 mg of the total enzymatic fiber based on the combined percentage of insoluble and soluble fractions from the Hellendoorn analyses (8,9). This quantity was treated sequentially at 37 C under N_2 with magnetic stirring as follows: (1) 3 mg pepsin, pH 1.5, 3 hr; (2) 3 mg glucoamylase plus 6 mg pancreatin, pH 6-6.5, 3 hr; (3) 6 mg hemicellulase plus 0.1 mg pectinase, pH 6.9-7.1, 5 hr; (4) pH 7.1-7.2, 14 hr. The sample was cooled to room temperature for each pH adjustment and a drop of $CHCl_3$ was added each time to retard spoilage. Residual $CHCl_3$ was removed by heating the mixture at 40 C for 5 min in a stream of N_2.

For the binding determination of individual enzymatic insoluble or soluble fractions from banana and guava, the first 2 steps were omitted (pepsin, pancreatin and glucoamylase). The sample (20 mg) was mixed with 3.0 ml 0.85% NaCl and the pH adjusted to 6.9-7.1 with 0.1 N HCl and 1% NaOH. Hemicellulase and pectinase were added as described above and the remainder of the procedure was carried out.

Cholesterol Binding Procedure

A mixture of 1.7 mg cholesterol, 1 mg triolein, 4 mg palmitoyl lysolecithin, 1 mg deoxycholic acid, 1.1 mg cholic acid, 2 mg sodium taurocholate, 3 mg monolein and 6 mg oleic acid in a mixture of 2 ml tetrahydrofuran and 1 ml methanol was placed in a centrifuge tube and the solvent was evaporated in a nitrogen stream. The degraded fiber sample, suspended in 3 to 4 ml of solution, was poured into the centrifuge tube containing the lipids. Approximately 0.5 ml of additional 0.85% NaCl solution was used to wash a small amount of residual insoluble material into the centrifuge tube. The tube was then placed in a sonic bath (Cole-Parmer Model 8845-30) for 5 min. The resultant cloudy mixture appeared to be free of any residual insoluble lipids on the tube walls. A 10 mg sample of tribasic calcium phosphate [$Ca_{10}(OH)_2(PO_4)_6$] was added and the tube stirred magnetically under N_2 for 1 hr at 37 C. The stirring bar was removed and washed with a small amount of ether. The tube was centrifuged for 5 min at 1000 g and the supernatant decanted. The walls in the upper part of the tube and the top of the residue were washed with 1-2 ml of ether. The supernatant was dried by evaporating the water in a Rotavap at 40 C/15 torr, then evacuated to 1 torr at 40 C. The combined washings from the stirring bar and tube were diluted to 10 ml with

ether and the ether solution was used to extract the dried supernatant. The centrifuge residue was dried by evacuation at 40 C/15 torr, then at 40 C/1 torr. Cholesterol was extracted from the 2 pulverized dried products with 3 or 4 portions of ether (total extract volumes 10 ml each).

Cholesterol was determined in the ether extracts by high pressure liquid chromatography (HPLC). A 100 μl sample of the filtered extract (fine sintered glass filter) was injected. The column was a Varian MCH-10 (10 μ C-18, 4 mm i.d. x 30 cm length). Peaks were detected by UV at 213 nm. The solvent mixture was 79% acetonitrile-20% tetrahydrofuran-1% methanol. At a flow rate of 2 ml/min, the retention time of cholesterol was 9 min. The cholesterol peak was quantitated by comparing the peak area (planimeter) with those of standard cholesterol solutions. The peak area-concentration relationship was linear in the concentration ranges observed for these samples.

RESULTS AND DISCUSSION

The cholesterol binding capacity of 28 fiber samples from tropical fruits and vegetables was measured by a modified in vitro method. The capacities of commercial cholestyramine, cellulose, lignin, guar gum and citrus pectin were also determined.

The method includes an enzymatic pretreatment designed to simulate the effect of the human digestive system on the fiber. In the first two steps, the procedure is similar to the Hellendoorn enzymatic analysis (treatment with pepsin, glucoamylase and pancreatin). The resulting product has been degraded much like fiber that has traveled through the stomach and upper intestine. In the third step (hemicellulase and pectinase treatment), the fiber was further degraded by enzymes similar to those produced by microorganisms in the large intestine. Finally, the degraded fiber was mixed with a mixture containing cholesterol that is representative of the lipid composition in the large intestine, and centrifuged. The residue contained the insoluble fiber and associated cholesterol, along with the insoluble calcium phosphate. The supernatants contained a cloudy dispersion indicating the presence of micelles. HPLC analysis of an ether extract of the aqueous supernatants showed that only a small fraction of the cholesterol could be recovered from this dispersion. Because the presence of water reduced the extraction efficiency, the amount of cholesterol in the centrifuge residue and supernatant was determined by extracting the dried products. Binding values were calculated as the percentage of total cholesterol in the residue. The resulting values should be representative of the binding capacities of the fiber residue for cholesterol and similar lipids in the lower digestive tract.

Model experiments were carried out with cellulose (Solka-Floc), the mixture of 5 enzymes and calcium ion ($CaCl_2$) or insoluble calcium in the form of tribasic calcium phosphate [$Ca_{10}(OH)_2(PO_4)_6$]. When $CaCl_2$ was the calcium source, over 90% of the cholesterol was present in the residue. When tribasic calcium phosphate was used or no calcium was added, about 20% of the cholesterol was in the residue. Since most of the calcium in human feces is present as insoluble calcium phosphate, the conclusion was made that calcium should be added as insoluble calcium phosphate rather than as calcium ion.

Fecal pH in certain ethnic groups can be as low as 5.7 (11). Since more acidic conditions can increase calcium ion concentration, a model experiment was conducted with tribasic calcium phosphate added and the pH of the final step adjusted to 5.7 instead of 7.1-7.2. No difference in the amount of cholesterol in the residue was observed. Any increase in calcium ion concentration resulting from the increased acidity was not large enough to affect the binding capacity.

Further model experiments were carried out with cellulose, the enzyme mixture and calcium phosphate. Eliminating either the enzyme mixture or tribasic calcium phosphate did not significantly change the binding value. With enzymes and calcium phosphate present and no cellulose, the amount of cholesterol in the residue was insignificant. Thus, the presence of the enzymes, insoluble tribasic calcium phosphate, or a combination of the two did not, by themselves, appear to influence the binding value.

As Table 1 shows, only fiber from sweet potato flesh, carambola and Keitt mango had a greater binding capacity than cellulose or lignin. Even these samples did not approach the capacity of cholestyramine. Samples with high lignin concentrations, such as sweet potato peel and date, did not have a significantly higher capacity.

The capacity of many of the samples was significantly below that of cellulose. Coconut fiber had a very low capacity. Since coconut fiber contains a large hemicellulose fraction (9), extensive hemicellulose hydrolysis could account for its poor binding properties. The hydrolysis of pectin could explain the low binding affinities of citrus pectin and kiwi fiber (which contains a large pectin fraction). The

lowest capacity was shown by fiber from the African yam, *Dioscorea rotundata.* This sample contained the highest concentration of enzymatic soluble fiber, 4.1% of the fresh wt (9). This result suggests that the binding ability of the soluble fractions may be relatively low.

Considering the capacity per unit of lignocellulose (lignin + cellulose [8,9]), as shown in the right-hand column of Table 1, reveals considerable variation. For most of the samples, the values are greater than for lignin or cellulose. By making two assumptions: 1) hemicellulose and pectin are completely degraded, and 2) the lignocellulose residue has about the same capacity in all samples, these capacity differences could be explained by the presence of other stable fiber components (such as gums or mucilages). Fiber from *D. rotundata* was unusual because it had the highest capacity per unit of lignocellulose (6.9) combined with a very low total capacity (7%). In this case, the capacity of the other stable component must be very large compared with lignocellulose.

The capacities of individual enzymatic fractions from 3 of the more economically important samples (sweet potato, cassava and grapefruit) were determined. In each case, the value for the soluble fraction was much lower than that of the insoluble fraction (Table 1). An exceptionally low value was shown by the soluble fraction from cassava (3%). Since the soluble fraction, in most cases, is composed of soluble pectin and hemicellulose, the low values are probably the result of enzymatic hydrolysis. The total capacity, calculated by assuming that the contribution of each fraction was proportional to its relative amount, was significantly less than the capacity determined from the corresponding AIR.

The mean value for unrecovered cholesterol, calculated by subtracting the sum of the amounts in the ether extracts of the supernatant and residue from the total cholesterol, was zero for the fruits and 33% for the starchy vegetables. The dried supernatants from the starchy vegetables were viscous liquids that undoubtedly contain large amounts of glucose derived from the hydrolysis of the starch. Apparently, cholesterol was difficult to recover from this residue by ether extraction, and the result was a high value for unrecovered cholesterol.

In general, the binding ability of most samples was equal to or less than cellulose or lignin. In contrast, many feeding studies have shown that the hypocholesteremic effect of pectin and guar gum is between the effect of cellulose and cholestyramine. Our cholesterol binding data do not appear to support the conclusion from in vivo studies that the direct binding of sterols or bile acids in the large intestine is responsible for the reduced blood cholesterol.

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REFERENCES

1. Kay, R.M. (1982) J. Lipid Res. 23, 221-242.
2. Vahouny, G.V. (1982) Fed. Proc. Fed. Am. Soc. Exp. Biol. 41, 2801-2806.
3. Story, J.A. (1981) in Advances in Lipid Research (Paoletti, R., and Kritchevsky, D., eds.) Vol. 18, pp. 229-246, Academic Press, New York.
4. Vahouny, G.V., Tombes, R., Cassidy, M.M., Kirtchevsky, D., and Gallo, L.L. (1980) Lipids 15, 1012-1018.
5. Prema, P., and Kurup, P.A. (1979) Indian J. Exp. Biol. 17, 1341-1345.
6. Salyers, A.A. (1979) Am. J. Clin. Nutr. 32, 158-163.
7. Cummings, J.H. (1982) in Dietary Fiber in Health and Disease (Vahouny, G.V., and Kritchevsky, D., eds.) pp. 9-22, Plenum Press, New York.
8. Lund, E.D., and Smoot, J.M. (1982) J. Agric. Food Chem. 30, 1123-1127.
9. Lund, E.D., Smoot, J.M., and Hall, N.T. (1983) J. Agric. Food Chem. 31, 1013-1016.
10. Shipley, E.A. (1978) in Topics in Dietary Fiber Research (Spiller, G.A., and Amen, R.J., eds.) pp. 203-212, Plenum Press, New York.
11. Hellendoorn, E.W. (1978) in Topics in Dietary Fiber Research (Spiller, G.A., and Amen, R.J., eds.) p. 158, Plenum Press, New York.

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Daily Variations of the Biosynthesis and Composition of Fatty Acids in Rats Fed on Complete and Fat-Free Diets

I.N.T. DE GÓMEZ DUMM,[1] M.J.T. DE ALANIZ[1] and R.R. BRENNER,[1]* *Instituto de Investigaciones Bioquímicas de La Plata (INIBIOLP), CONICET-UNLP, Facultad de Ciencias Médicas, calles 60 y 120, 1900-La Plata, Argentina*

ABSTRACT

This report describes the daily changes in fatty acid composition and fatty acid desaturation in rats feeding on a complete diet and a fat-free diet successively. Rats on a complete diet showed a good homeostasis in the percentage of fatty acid in plasma, with a possible palmitic acid rhythm, but the fat-free diet initiated an essential fatty acid-deficient pattern in a few hours. The light-dark period in animals feeding on a complete diet motivates a feeding rhythm that causes changes in linoleic and arachidonic acids in the whole liver and microsomes that are related to Δ6 and Δ5 desaturase activities. The patterns of Δ6 and Δ5 desaturase changes were different. Linoleic acid intake during the dark periods (complete diet feeding) caused a decrease of Δ6 desaturase activity and the activation of Δ5 desaturation that led to an increase of arachidonic acid biosynthesis. The feeding of a fat-free diet eliminated the rhythm observed in linoleic and arachidonic acid composition in the liver and changed the desaturase rhythms. The Δ9 desaturase activity in the liver also showed a daily rhythm in the complete-diet period that disappeared with the change to a fat-free diet, while the activity increased markedly. A negative correlation existed between the percentage of linoleic acid in the liver and the Δ9 desaturase activity. However, no correlation was found between Δ9 desaturase activity and the percentage of 16:1 and 18:1 in the complete-diet period.
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INTRODUCTION

Unsaturated fatty acid biosynthesis is believed to be controlled mainly by the regulation of microsomal desaturase activity that occurs at the Δ9, Δ6 and Δ5 positions in the fatty acid chains (1-3). Desaturation at these positions is catalyzed by specific enzymes subject to diurnal changes (4). These diurnal fluctuations have also been observed in lipid composition and in several enzymes related to lipid metabolism in rodents (4-8).

Circadian rhythms have been also demonstrated in protein synthesis (4), blood sugar (9), insulin (9), glucagon (10) and corticoids (11). Some good evidence exists that these factors, as well as food composition, may modify unsaturated fatty acid biosynthesis (12-15).

To obtain further information on the day and night mechanisms ruling the oscillations of unsaturated fatty acid biosynthesis, the Δ9, Δ6 and Δ5 fatty acid desaturase activity of rat-liver microsomes was measured periodically in animals feeding successively on a complete and a fat-free diet. The results were compared with the changes in the fatty acid composition of the whole liver, liver microsomes, plasma and epididymal fat tissue.

*To whom correspondence should be addressed.

[1] Members of the Carrera del Investigador Científico, Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina.

MATERIALS AND METHODS

Chemicals

[1-^{14}C]Palmitic acid (56 mCi/mmol) and [1-^{14}C]linoleic acid (58 mCi/mmol) were purchased from the Radiochemical Centre, Amersham, England. [1-^{14}C]Eicosa-8,11,14-trienoic acid (54.7 mCi/mmol) was purchased from New England Nuclear Corp., Boston, MA. Tests for radiochemical purity were made in our laboratories and the materials were ca. 98-99% pure. Unlabeled fatty acids were purchased from NuChek Prep, Inc., Elysian, MN.

Animals and Their Treatment

Male Wistar rats were kept in rooms at a temperature of 22 ± 1 C and given water and food ad libitum (Purina Chow). Light (fluorescent white light 40W), from 08:00 to 20:00 hr, alternated with 12 hr of darkness. When the rats were 21 days old, they were weaned and caged in groups of 4 until they were sacrificed. When two months old, 7 groups of 4 animals were killed by decapitation at 4 hr intervals during a 24 hr period. At this time, the food of the remaining 6 groups of animals was changed to a fat free diet. The rats were then sacrificed by groups at 4 hr intervals during a 24 hr period. Purina Chow consisted of : (in calories) 56.7% carbohydrates, 10.4% lipids and 32.9% protein, vitamins and minerals. The fatty acids of this diet included 21.4% palmitic, 2.1%

palmitoleic, 8.2% stearic, 24.9% oleic, 37.7% linoleic and 0.2% arachidonic acids. The fat-free diet consisted of: (in calories) 73.4% starch and 26.6% defatted casein, supplemented with minerals (16) and a mixture of vitamins (17). Four animals were studied individually at each time. The blood was allowed to drain and was collected in heparinized tubes to measure fatty acid composition.

The livers were rapidly excised and immediately placed in an ice-cold homogenizing medium as described by Castuma et al. (18). After homogenization, samples were taken to measure protein content and fatty acid composition. Liver microsomes were separated by differential centrifugation at 100,000 g as described elsewhere (18). Aliquots of liver microsomes were analyzed for protein content and fatty acid composition. The epididymal fat pad was also analyzed for fatty acid composition.

Incubation Procedure for Oxidative Desaturation of Fatty Acids

Desaturation of the fatty acids by liver microsomes was measured by estimating the percentage conversion of [1-^{14}C]palmitic acid to palmitoleic acid, [1-^{14}C]linoleic acid to γ-linolenic acid and [1-^{14}C]eicosa-8,11,14-trienoic acid to arachidonic acid. Three nmol of labeled acid and 97 nmol of unlabeled acid were incubated with 5 mg of microsomal protein in a metabolic shaker at 37 C for 10 min, in a total volume of 1.5 ml of 0.15 M KCl-0.25 M sucrose solution. The medium contained 4 μmol of ATP, 0.1 μmol of CoA, 1.25 μmol of NADH, 5 μmol of $MgCl_2$, 2.25 μmol of glutathione, 62.5 μmol of NaF, 0.5 μmol of nicotinamide, and 62.5 μmol of phosphate buffer (pH 7).

Analysis of Radioactive Fatty Acids

Incubations were stopped by the addition of 2 ml of 10% KOH in methanol. The fatty acids were recovered by the saponification of the incubation mixture. The acids were esterified with 3 M HCl in methanol (3 hr at 68 C). The distribution of radioactivity between the different fatty acids was determined by gas-liquid radiochromatography in an apparatus equipped with a Packard proportional counter using the procedure described previously (18). The labeled methyl esters were identified by equivalent chain length determination and by comparing them with authentic standards.

Analysis of Fatty Acid Composition

In order to determine the fatty acid composition of the different tissues, lipids were extracted with chloroform/methanol (2:1, v/v) by the procedure of Folch et al. (19). Fatty acids were converted to methyl esters and analyzed by gas-liquid chromatography in a Hewlett-Packard apparatus, Model 5840 A. The column was packed with 15% EGSS-X coated on Chromosorb WHP (80-100 mesh), Supelco Inc., Bellefonte, PA. The fatty acids were identified by comparing them with standards.

Analytical Determinations

Protein was determined by the biuret method of Gornall et al. (20).

RESULTS

Plasma

Figure 1 illustrates the daily fluctuations in the relative concentration of fatty acids in the plasma lipids of rats fed Purina Chow for 24 hr followed by 24 hr on a fat-free diet. In spite of the homeostasis, different minor daily fluctuations of plasma lipids are observed in animals fed a complete diet. The study of plasmatic essential fatty acids (Fig. 1-A) revealed that linoleic acid showed a high and rather constant level during the first day, but declined during the 24 hr period of fat deprivation. At the end of the experiment, the percentage of plasma linoleic acid was ca. one-third of the initial values. The level of arachidonic acid followed a rather similar fluctuation, remaining high and practically constant during the feeding with a complete diet but dropping with the fat-free diet.

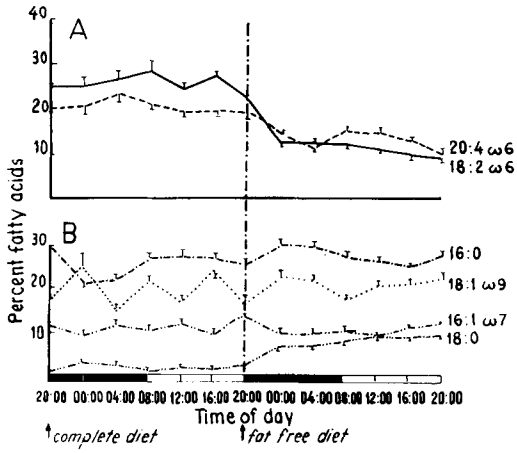

FIG. 1. Daily fluctuations in the relative concentration of fatty acids in the plasma lipid of rats fed for 24 hr on a complete diet, followed by 24 hr on a fat-free diet. Arrows indicate the time each diet was administrated. The black line on the abscissa represents the dark periods. At each time-point, the values represent the mean ± SEM of 4 animals.

In the nonessential fatty acid group (Fig. 1-B), stearic and oleic acids showed some rapid fluctuations during the period of complete diet that were apparently nonrhythmical. Palmitic acid showed a transient decline during the first night period between 20:00 and 04:00 hr whereas palmitoleic acid remained practically constant. The fat deprivation in general smoothed the curves, eliminated the negative night peak of palmitic acid and evoked a progressive increase of palmitoleic acid. Oleic acid, after a night peak, also increased steadily.

Liver: Essential Fatty Acids

The essential fatty acid composition of total lipids in the liver showed daily fluctuations (Fig. 2-A). The percentage of linoleic acid increased remarkably at night during the feeding period, reaching its maximum at 04:00, then showing a steady decline. The lowest value was found at the end of the resting period. Arachidonic acid also began to increase at the onset of night. The highest level was reached during the light period with a decline at the end of daylight. The oscillation of arachidonic acid seems to be displaced in relation to linoleic acid changes.

Daily fluctuations in the percentage of linoleic and arachidonic acid were also shown in the liver microsomal membrane of the rats fed a complete diet (Fig. 2-A). However, linoleic acid oscillations were less pronounced in microsomes than in the total liver. The ascending slope of arachidonic acid in microsomes preceded that observed in the total organ. The change to a fat-free diet and the absence of linoleic acid in the food of the rat caused the linoleic acid daily variations to disappear and both essential fatty acids, linoleic and arachidonic, declined steadily in the liver (Fig. 2-A). However, the decline of arachidonic acid was later than that of linoleic acid. With this diet, the correlation between the percentage of each acid in the whole liver and in microsomal membrane (correlation coefficient 0.97 for both acids) was perfect.

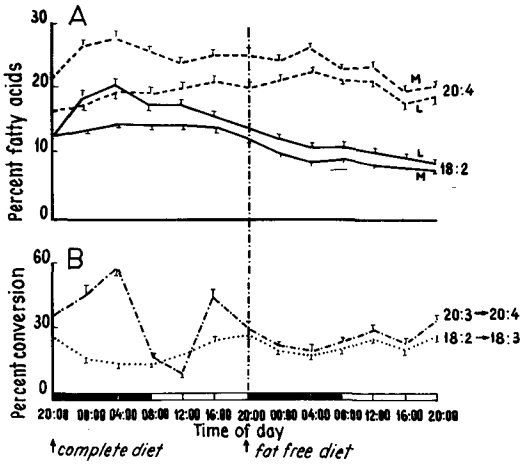

FIG. 2. Daily changes of Δ6 and Δ5 desaturation activity and the relative composition of essential fatty acids in the liver. A. Percentage of linoleic (———) and arachidonic acid (– – – –) in the total liver homogenate (L) and liver microsomes (M). B. Percentage of conversion of linoleic acid to γ-linolenic acid (.....) and eicosa-8,11,14-trienoic acid to arachidonic acid (–.–.–.). Id. Fig. 1.

Figure 2-B illustrates the changes of Δ6 and Δ5 desaturation activity in the liver microsomes of male rats fed a complete diet followed by a fat-free diet. The Δ6 desaturase exhibited a diurnal variation as has been shown in female mice (4). The present experiment also showed that the Δ5 desaturase activity changes rhythmically, though the phases of the 2 desaturase oscillations were different. The Δ6 desaturase showed a decline in the conversion during the night while the Δ5 desaturase increased. The Δ6 desaturase increase was produced at the onset of the light period and was followed by a second peak of the Δ5 desaturase. Maximum Δ6 desaturase activity was at 20:00 hr, when the linoleic acid concentration in the liver reached its lowest value. The decline of the Δ6 desaturase during the dark period was correlated with the increase of linoleic acid concentration in liver microsomes (correlation coefficient $R = 0.92$, regression coefficient: $a = 100$ and $b = -5.97$).

The administration of the fat-deficient diet synchronized the fluctuations of both desaturases with an evident decrease in Δ5 conversion. Both enzymes showed a small peak at noon ($p < 0.05$) and a small increase at the end of 24 hr of fat deficiency.

Liver: Nonessential Fatty Acids

Figure 3 illustrates the fluctuations of the saturated and monoenoic fatty acids and the Δ9 desaturase activity changes in the livers of rats fed a complete diet followed by a fat-free diet. The whole liver and microsomes showed similar fatty acid compositions (Fig. 3-A). The curves in both biological materials showed a perfect fitness with correlation coefficients in the range of 0.96 to 0.99. When the rats were on a complete diet, a decrease in the percentage of palmitic acid in liver was observed, with a minimum at 04:00 hr. This fluctuation of palmitic acid has been already shown in mice (4). No detectable daily changes could be recognized for the other nonessential fatty acids. During fat deprivation, palmitic, palmit-

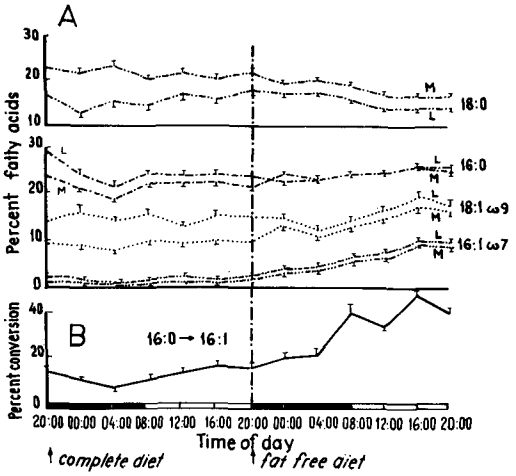

FIG. 3. Relative concentration of saturated and monoenoic fatty acids and Δ9 desaturation activity in the liver. A. Percentage of stearic acid (–··–··–··), palmitic acid (–·–·–·), oleic acid (.....) and palmitoleic acid (–···–···–) in total liver homogenate (L) and in liver microsomes (M). B. Percent conversion of palmitic to palmitoleic acid (———). Id. Fig. 1.

oleic and oleic acids increased continuously whereas stearic acid tended to decline. Daily changes also occurred in Δ9 desaturation of palmitic to palmitoleic acid (Fig. 3-B). These changes were similar to those found in mice (4), decreasing at the onset of the night and beginning to increase at the end of the dark period, the maximum was reached at the end of the light period. The absence of fat in the diet activated the Δ9 desaturase steadily.

Epididymal Fat Pad

The fluctuations in the percentages of fatty acids in the epididymal fat pad were not represented because they were much smaller than those observed in the plasma and liver. The most important change was observed in linoleic acid. It showed a peak at the onset of the night, when the rats were fed a complete diet, and decreased significantly when the animals were given a fat-free diet. No variations in the other fatty acids were recognized.

DISCUSSION

Rats eat mostly at night and hardly at all during the day (6). Since the complete diet provided linoleic acid as the only practical source of essential fatty acids, the arachidonic acid present in the animal must be produced by biosynthesis. Other acids, such as palmitic, stearic, oleic and palmitoleic, may have a dual origin—exogenous and endogenous. Palmitic acid may be formed from acetate by de novo synthesis; stearic acid can be synthesized from palmitic by elongation; oleic and palmitoleic acids can be formed from stearic and palmitic acids, respectively, by Δ9 desaturation. Elongation and desaturation reactions are produced in the endoplasmic reticulum whereas de novo biosynthesis is produced in the cytosol.

The oscillation of fatty acid composition of plasma lipids during the day is undoubtedly the compromise of all the processes outlined before, together with the mobilization from adipose tissue and liver, oxidation and incorporation in the tissue. The balance of these metabolic processes is shown by the small changes in the percentages of fatty acids in the plasma of rats feeding a complete diet where only the fluctuations of palmitic acid may represent a circadian rhythm.

When the rats ate a fat-free diet, the general decline of linoleic and arachidonic acids and the increase of palmitoleic acid indicated an incipient essential fatty acid deficiency (Fig. 1).

The composition of essential fatty acids in the liver and microsomes showed that when the rats were fed a complete diet, food intake increased the linoleic acid percentage in the liver markedly during the dark period and enhanced arachidonic acid biosynthesis, causing a rapid increase in the microsomes and a later accumulation of this acid in the tissue (Fig. 2-A). Therefore, the increase of arachidonic acid during the first night could be explained by the increase of linoleic acid in the liver and by the highest conversion of eicosatrienoic acid to 20:4 acid during this period (Fig. 2-B). An excellent positive correlation between the ascending part of Δ5 desaturase peak and 18:2 percentage of liver microsomes was found during the considered period ($R = 1.00$, $a = 100.3$, $b = 11.3$).

The Δ6 desaturase activity declined during the night, showing the existence of an inverse proportional ratio between linoleate and arachidonate concentration in microsomal membrane and Δ6 desaturation activity. These results are consistent with the linoleate and Δ6 desaturase relationship found in mice microsomes (4). In connection with these results, an essential fatty acid deficiency generally increases Δ6 linoleate desaturation (21), an effect that is reversed by feeding linoleate (22).

A high-fat diet rich in linoleic acid has been shown to stimulate the activity of hepatic microsomal Δ5 desaturase (23) whereas an essential fatty acid deficient diet decreases the Δ5 desaturase activity of liver (22). Therefore, the Δ6 and Δ5 desaturases fluctuations and the activation of the eicosa-8,11,14-trienoic acid

conversion to arachidonic acid found in the experiment might be produced by the increased incorporation of linoleic acid into the liver in spite of a decrease of Δ6 desaturase activity. The results obtained during the first night would also imply that enough 20:3ω6 acid is produced from linoleic acid, even with the decrease of Δ6 desaturase activity. However, the second peak of Δ5 desaturation activity is found at the end of the light period when linoleic acid is already declining. Therefore, linoleic acid concentration would seem not to be the only possible factor that may regulate this conversion. Moreover, the small peak found for Δ5 and Δ6 desaturases at 12:00 hr during the fat-free period cannot be explained by a linoleic acid rhythm in the liver.

A circadian rhythm of the Δ9 desaturase has been shown in the female mouse liver (4). During the first 24 hr of the present experiment, when male rats ate a complete diet, the Δ9 desaturation of palmitic to palmitoleic acid also showed daily variations (Fig. 3-B). In the rat, the Δ9 desaturase fluctuations coincided with the Δ6 desaturation oscillations during this period, and were thereby out of phase with Δ5 desaturation changes. The fluctuations had a negative correlation to the linoleic acid of the liver (Figs. 2 and 3). The lowest activities of Δ9 and Δ6 desaturases corresponded to the lowest linoleic acid percentage and no good correlation was found with other liver fatty acids. Therefore, the results agree with Jeffcoat and James' (23) demonstration that dietary linoleic acid represses Δ9 desaturase. Moreover, the absence of linoleic acid in the diet produced a progressive increase in the percentage conversion of palmitic acid to palmitoleic acid, that conforms to previous works that demonstrated an activation of the Δ9 desaturase by a fat-free diet (23,24). In consequence, the variations of the Δ9 desaturation observed in the present experiment could be explained by a modulation effect in linoleic acid ingestion. This interpretation is strengthened by the disappearance of the Δ9 desaturase changes when the rats were fed a fat-free diet.

In conclusion, comparing the daily variations of fatty acids found in animals feeding on a complete diet with those obtained with a fat-free diet, injection of the fat-free diet rapidly triggers the development of an essential fatty acid deficiency. At the same time, the typical changes of linoleic, arachidonic and nonessential fatty acids, as well as the Δ9, Δ6 and Δ5 desaturase fluctuations of the liver disappear or are modified. In consequence, dietary fat is a triggering factor in the daily changes. Also, since nonessential fatty acids are easily synthesized by the animal, dietary linoleic acid would be the only absolute exogenous fatty acid in the complete diet. Therefore, we postulate that linoleate probably provokes circadian changes of the desaturases, but other factors probably modify the linoleic acid effect as well.

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REFERENCES

1. Brenner, R.R. (1974) Mol. Cell. Biochem. 3, 41-52.
2. Ullman, D., and Sprecher, H. (1971) Biochim. Biophys. Acta 248, 186-197.
3. Brenner, R.R. (1977) Drug. Metab. Rev. 6, 155-212.
4. Actis Dato, S.M., Catalá, A., and Brenner, R.R. (1973) Lipids 8, 1-6.
5. Hickman, P.E., Horton, B.J., and Sabine, R.J. (1972) J. Lipid Res. 13, 17-22.
6. Kimura, T., Maji, T., and Ashida, K. (1970) J. Nutr. 100, 691-697.
7. Edwards, P.A., Mureya, H., and Gould, R.G. (1972) J. Lipid Res. 13, 396-401.
8. Actis Dato, S.M., and Brenner, R.R. (1974) Acta Physiol. Latinoam. 24, 199-203.
9. Jarrett, R.J. (1979) in Comprehensive Endocrinology, Series Endocrine Rhythms (Krieger, D.T., ed.), pp. 247-258, Raven Press, New York.
10. Gagliardino, J.J., Pessacq, M.T., Hernández, R.E., and Rebolledo, R.O. (1978) J. Endocr. 78, 297-298.
11. Turton, M.B., and Deegan, T. (1974) Clin. Chim. Acta 55, 389-397.
12. Peluffo, R.O., Gómez Dumm, I.N.T. de, Alaniz, M.J.T. de, and Brenner, R.R. (1971) J. Nutr. 101, 1075-1084.
13. Gómez Dumm, I.N.T. de, Alaniz, M.J.T. de, and Brenner, R.R. (1975) J. Lipid Res. 16, 264-268.
14. Gómez Dumm, I.N.T. de, Alaniz, M.J.T. de, and Brenner, R.R. (1979) J. Lipid Res. 20, 834-839.
15. Gómez Dumm, I.N.T. de, Alaniz, M.J.T. de, and Brenner, R.R. (1970) J. Lipid Res. 11, 96-101.
16. Rogers, Q.R., and Harper, A.E. (1965) J. Nutr. 87, 267-273.
17. Peluffo, R.O., Brenner, R.R., and Mercuri, O. (1963) J. Nutr. 81, 110-116.
18. Castuma, J.C., Catalá, A., and Brenner, R.R. (1972) J. Lipid Res. 13, 783-789.
19. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497-509.
20. Gornall, A.G., Bardawill, C.J., and David, M.M. (1949) J. Biol. Chem. 177, 751-766.
21. Peluffo, R.O., Nervi, A.M., and Brenner, R.R. (1976) Biochim. Biophys. Acta 441, 25-31.
22. Brenner, R.R. (1981) in Essential Fatty Acids and Prostaglandins (Holman, R.T., ed.) pp. 41-47, Pergamon Press, New York.
23. Jeffcoat, R., and James, A.T. (1977) Lipids 12, 469-474.
24. Paulsrud, J.R., Stewart, S.E., Graff, G., and Holman, R.T. (1970) Lipids 5, 611-616.

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Iodothyronines: Oxidative Deiodination by Hemoglobin and Inhibition of Lipid Peroxidation[1]

YUEH-CHU L. TSENG[2] and KEITH R. LATHAM*, *Department of Medicine, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814*

ABSTRACT

Purified rat hemoglobin catalyzes the oxidative degradation of iodothyronines to form iodide and an iodine-containing intermediate that reacts with protein. Hemoglobin also catalyzes peroxidation of linoleic acid. These observations are consistent with the reported intrinsic peroxidase activity of hemoglobin and other heme-proteins. However, incubations containing both linoleic acid and an iodothyronine produced a surprising result: deiodination was stimulated rather than competitively inhibited. In contrast, linoleic-acid peroxidation was inhibited by iodothyronines. Thus, low levels of iodothyronines (2.6×10^{-7}M) are effective inhibitors of linoleic-acid peroxidation. Thyroxine and reverse T_3 were found to be more effective in this antioxidant activity than vitamin E, glutathione, ascorbic acid and DTT. Since linoleic-acid peroxidation proceeds by a propagating free-radical mechanism, we have concluded that iodothyronines can effectively terminate the free-radical chain reaction to become oxidatively deiodinated. Consistent with this antioxidant mechanism, reverse T_3 is effective in preserving red cell membranes as measured by the inhibition of erythrocyte hemolysis.
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INTRODUCTION

In our previous study of reductive bovine-liver deiodinases (1), in which detergent-solubilized membrane proteins were fractionated by column chromatography, we consistently observed a deiodinase activity that coeluted with contaminating hemoglobin. In contrast to the reductive deiodinases, this activity was stimulated by the addition of soybean phospholipid preparations that contained high levels of unsaturated fatty acids. By a number of measures, we identified this activity with hemoglobin. Hemoglobin has been reported to undergo slow autoxidation to generate superoxide in a reaction that oxidizes epinephrine to form adrenochrome (2). Hemoglobin also catalyzes the oxidation of unsaturated fatty acids, forming unstable free radicals as intermediates (3,4). In the present study, we investigated the mechanism by which hemoglobin catalyzes both iodothyronine deiodination and the oxidation of unsaturated fatty acids. In addition, we investigated the possible role of iodothyronines as antioxidants and free-radical scavengers.

[1] The opinions or assertions contained here are the authors' and are not to be construed as official or as reflecting the views of the Department of Defense or the Uniformed Services University of the Health Sciences. The experiments reported here were conducted according to the principles in the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council DHEW Pub. No. (NIH) 74-23.

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* To whom correspondence should be addressed.

MATERIALS AND METHODS

Preparation of Purified Hemoglobin

Blood from female Sprague-Dawley rats (4 to 6 mo) was obtained by cardiac puncture with a heparin-rinsed syringe. The blood was centrifuged, the buffy coat removed, and the erythrocytes washed 3 times with 3 vol of 0.15 M saline. The washed erythrocytes were lysed in 4 vol of distilled water, centrifuged (50,000 × g, 20 min) at 4 C and the clear erythrocyte lysate stored in liquid nitrogen for further use. Hemoglobin was purified by gel-exclusion chromatography: 1 ml of erythrocyte lysate was loaded onto a Sephacryl S-200 column (Pharmacia, 1.6 × 90 cm) equilibrated with an assay buffer (15 mM potassium phosphate, pH 7.2, 0.32 M sucrose, 1 mM EDTA, 6 mM magnesium chloride), and 2.0 ml fractions were collected at a flow rate of 0.16 ml/min. The hemoglobin peak was identified visually and quantitated by optical absorbence at 414 nm (5). Protein concentrations were measured by a dye-binding method (6).

Lipoxidase Assay

Using linoleic acid as a substrate, the increased absorption at 234 nm during linoleic-acid peroxidation was measured; the change in absorbency between zero time and 5 min ($\Delta A_{5\ min}$) was used as an index of lipoxidase activity (7). To prepare a stock substrate solution, 10 μl of linoleic acid (99% pure, Sigma Chemicals) was dissolved in 6 ml absolute alcohol and diluted to 10 ml in water with thorough mixing by stirring in a closed vial.

Just before use, the stock solution was diluted with 0.2 M borate buffer (pH 9.0) to a final concentration of 2.6×10^{-4} M. The assay was initiated by adding 20 µl of purified (Sephacryl S-200) erythrocyte lysate hemoglobin to 980 µl of substrate solution and absorbence at 234 nm was recorded at 0 and 5 min.

This assay system was used to test the ability of various thyronines, reducing agents and antioxidants to inhibit hemoglobin-catalyzed oxidation of linoleic acid; 25 µl of antioxidant test solution was added to 955 µl of substrate solution, mixed thoroughly, then 20 µl (6 µg protein) of purified hemoglobin was added and the $\Delta A_{5\ min}$ at 234 nm was measured. Since linoleic acid slowly autoxidizes in air, the percentage of inhibition by each antioxidant was calculated relative to parallel incubations containing linoleic acid alone.

Erythrocyte Hemolysis Assay

Washed erythrocytes were diluted with 3 vol of 0.15 M NaCl and 75 µl of the cell suspension was added to 0.15 M NaCl (925 µl) containing 0.01% H_2O_2, in the presence or absence of 0.1 µM RT_3. The mixtures were incubated at 37 C for 30 min, then centrifuged to sediment erythrocyte ghosts and unhemolyzed cells. The hemoglobin concentration in the supernatant was estimated by absorbence at 525 nm (8).

Assay of Iodothyronine Deiodination

Aliquots of purified hemoglobin were tested for iodothyronine deiodination activity in incubations containing 4 nM outer-ring ^{125}I-labeled T_4, RT_3, and T_3 (New England Nuclear, Boston, MA) and various concentrations of the homologous unlabeled iodothyronines (Sigma Chemicals Co., St. Louis, MO). An aliquot (0.1 ml) of the incubation (35 C/20 min) was applied directly to a Sephadex G-25 superfine (Pharmacia) column for metabolite separation and quantitation as modified from Green (9). Sephadex G-25 superfine was equilibrated in column solution (0.1 N NaOH, 0.005 M NaCl), and columns were prepared with 2 different bed volumes depending on the resolution required for metabolite separation: 2 ml bed volumes were used for RT_3 incubations and 7 ml bed volumes were used for T_4 and T_3 incubations. The metabolites were eluted with column solution and the radioactivity in the various metabolite peaks was integrated. Parallel chromatograms from incubations containing only stock-radiolabeled hormones and buffer were used to subract the backgrounds of any minor contaminants in the standard. When contaminants were significant, radiolabeled thyronines were purified just before the experiment, on the Sephadex G-25, as described above. The column separations gave duplicate results with less than 1% variation on identical assays with over 95% recovery of the total radioactivity applied to the columns.

RESULTS AND DISCUSSION

Hemoglobin Purification: Demonstration of Deiodinase Activity

The fractions eluted from Sephacryl S-200 that contain hemoglobin were identified by the visible red color and were quantitated by absorbance at 414 nm. The elution position of the hemoglobin peak relative to standard proteins provided an estimated Stokes radius of 31.3 Å and a molecular weight of 50,000 Daltons. Partitioning of this relatively polar protein to the Sephacryl resin, retarding its elution, results in an apparent decrease in molecular weight relative to the reported 67,000 Daltons (10). The purified hemoglobin fraction has spectral absorption maxima at 577,540 and 414 nm, indicating properties identical to those reported for oxyhemoglobin (5).

Initially, two peaks of deiodinase activity (fr 36 and 39) were observed within the single hemoglobin peak (Fig. 1). This result was puzzling as we also observed that fractionation of erythrocyte lysate on Sephacryl S-200 in an

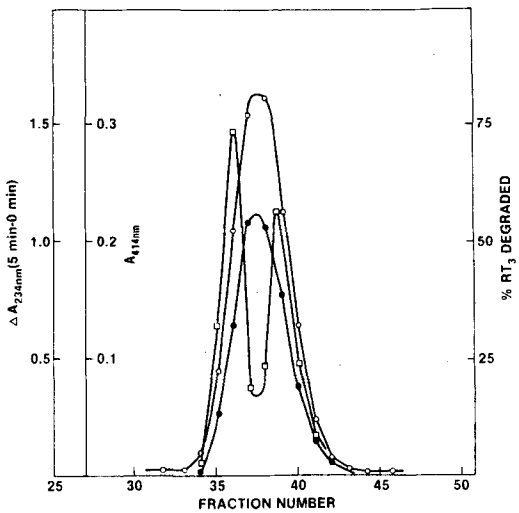

FIG. 1. Gel filtration of red blood cell lysate on Sephacryl S-200. Lysates were prepared as described in Methods; 1 ml (6 mg protein) was fractionated at 4 C (2 ml/fraction) on Sephacryl S-200 columns (Pharmacia, bed volume = 130 cc, flow rate = 0.16 ml/min). Individual fractions were assayed for RT_3 degradation (2 hr/35 C, □), hemoglobin concentration ($A_{414\ nm}$, •), and lipoxidase activity ($\Delta A_{234\ nm}$, ○).

assay buffer supplemented with 1 mM of the detergent CHAPS (to prevent protein aggregation) resulted in a single deiodinase peak, which precisely coeluted with hemoglobin. We subsequently found that the simple dilution of the active fractions in assay buffer (without detergent) also resulted in a single deiodinase peak that coeluted with the hemoglobin. We reasoned that hemoglobin was chelating most of the dissolved oxygen and that the apparent depression of deiodinase activity in the center of the eluted hemoglobin peak was the result of the lack of available oxygen, a required factor in the enzymatic process. Consistent with the requirement for oxygen in the reaction was our finding that 10 mM of the reducing agent dithiothreitol significantly inhibited the deiodination reaction. CHAPS probably modifies the hydrophobic environment around the heme group to reduce the affinity of hemoglobin for oxygen, resulting in more free oxygen in the reaction mixture.

Under our reaction conditions, 72% of the RT_3 metabolized appeared as inorganic iodide, 15% eluted with protein in the pass-through volume and 13% of the metabolites were unidentified; no detectable 3,3′-T_2 was generated. This absence of 3,3′T_2 generation contrasts with the quantitative production of 3,3′-T_2 from RT_3 by reductive deiodinases isolated from membranes (1,11) or the 5′ deiodination of thyroxine to T_3 (11-13). To examine the possibility that 3,3′-T_2 was a rapidly degraded intermediate, identical incubations were prepared that also included a 100-fold molar excess of unlabeled 3,3′-T_2; no radiolabeled 3,3′-T_2 accumulated. Incubations of purified hemoglobin fractions with other outer-ring ^{125}I-labeled iodothyronines (T_4, T_3, and 3,3′-T_2) showed predictable deiodination profiles based on our observations for RT_3. Radiolabeled inorganic iodide was the major metabolite, with labeled macromolecules as a minor product. Unfractionated erythrocyte lysate also deiodinated iodothyronines, but at only one-fifth the rate of Sephacryl-purified hemoglobin. Plasma showed negligible deiodination activity.

The radiolabeled macromolecular peak remained intact under the basic conditions (pH 12.5) of our Sephadex G-25 metabolite separation, suggesting the presence of a chemical bond between the proteins present and a radiolabeled, thyronine-derived component (14). Further characterization of this component by chromatography on Sephacryl S-200 column showed a major component of radiolabeled hemoglobin. Labeled proteins may be formed by direct peroxidase-catalyzed iodination using free iodide; alternatively, chemical bonding could occur between a reactively labeled iodothyronine intermediate and protein. However, the incubation of equivalent molar amounts of ^{125}I-iodide with hemoglobin resulted in a negligible amount of iodinated protein (compared with incubations with ^{125}I-RT_3). The labeled protein fraction is likely to be a protein-iodothyronine complex.

Determination of Km for Thyroxine Oxidative Deiodination by Hemoglobin

More detailed studies on the deiodination of T_4 by hemoglobin indicated that T_4 degradation was linear for up to 1 hr. Measurement of the initial velocities (up to 30 min) of deiodination at various substrate concentrations provided an estimated K_m of 0.13 μM for T_4 (Fig. 2). This value is close to that reported by Smallridge et al. for 3′,5′-T_2 deiodination by rat-liver cytosol (15).

Model for Iodothyronine Deiodination by Hemoglobin

Previous studies have shown that the oxidation of thyronine and its derivatives can result in the formation of chemically reactive semiquinone radicals and orthoquinones (16-18). Taken together, these results indicate that iodothyronine oxidation by hemoglobin probably results in the 3′- or 5′-hydroxylation of the thyronine beta-ring (Fig. 3, Scheme I) forming the ortho-hydroxy derivative (compound 2). Free iodide would be generated by the hydroxylation of an iodinated position. However, deiodination is not necessary for the formation of stable protein conjugates since T_3 and 3,3′-T_2 have only one outer-ring radiolabeled iodine, and these analogs also form labeled

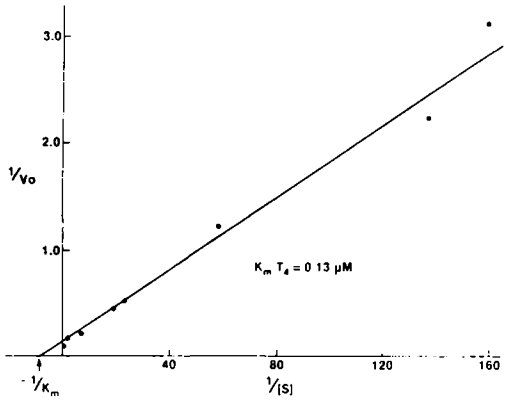

FIG. 2. Lineweaver-Burke plot of T_4 deiodination by purified rat hemoglobin. Thyroxine degradation rate was assayed at various substrate concentrations as described in Methods. (V_0, picomoles of T_4 degraded/mg protein/min; S, nanomolar concentration of T_4).

protein adducts. Protein conjugation probably occurs through the subsequent oxidation of the ortho-hydroxy groups to chemically reactive quinones (Fig. 3, compound 3). Inactivation of the quinone by other mechanisms probably occurs as an alternative to protein conjugation (18). Consistent with this model is the report of Plaskett (19) that incubation of T_4 with liver extract resulted in the formation of inorganic iodide and a partially deiodinated metabolite that was associated with protein. This metabolite-protein complex was stable under a variety of protein denaturing conditions and 3,5-diiodotyrosine (DIT) was released on tryptic hydrolysis. Production of DIT by ether cleavage following thyronine oxidation had been proposed previously as a pathway in the oxidation of thyronine by polyphenol oxidase (17).

Oxidation of Fatty Acids by Hemoglobin

An assay on the fractions eluted from Sephacryl S-200 for lipoxidase activity demonstrated a single activity peak that coeluted with hemoglobin (Fig. 1). This result confirmed the previous report that hemoglobin has intrinsic lipoxidase activity (3). This activity is known to proceed by a propagating free-radical mechanism that is initiated by the release of an allylic hydrogen radical, followed by an attack by oxygen on the generated carbon radical (3,4). The peroxide formed can generate another carbon radical and a hydroperoxide, thus propagating the chain until a terminating reaction occurs (3,4). Unsaturated fatty acids, which comprise about 43% of total fatty acids in red blood cell membranes (20), are particularly susceptible to oxidative damage. The reaction is activated by ferrous iron and hemoglobin (21). Vitamin E (α-tocopherol), the most important of the known antioxidants for protection against lipid peroxidation, is normally present in cell membranes at a molar ratio of 10^{-3} of vitamin E to polyunsaturated fatty acids (21).

Stimulation of Deiodination by Linoleic Acid

Incubation of 0.3 mM linoleic acid along with 4 nM ^{125}I-RT_3 and diluted hemoglobin at 35 C for 10 min resulted in a 3-fold increase in the rate of RT_3 degradation. The profile of products formed is similar to the unstimulated case, with inorganic iodide formed as a major product along with a minor radiolabeled macromolecular peak. The stimulation of RT_3 degradation by linoleic acid, rather than the expected competitive inhibition, suggested a mechanism in which linoleic acid was oxidized by hemoglobin to form the peroxide free radical that reacted with RT_3 (Fig. 3, Scheme II).

Antioxidant Effects of Iodothyronines

The addition of various iodothyronines to an incubation mixture containing hemoglobin and linoleic acid inhibited the peroxidation of the fatty acid (Table 1). Table 1 summarizes the antioxidation effects of iodothyronines and other antioxidants on linoleic-acid peroxidation by hemoglobin. Iodothyronines are more effective than α-tocopherol, ascorbic acid and glutathione in this antioxidant activity. Of the antioxidants examined, RT_3 has the highest activity. Although not measured in the present

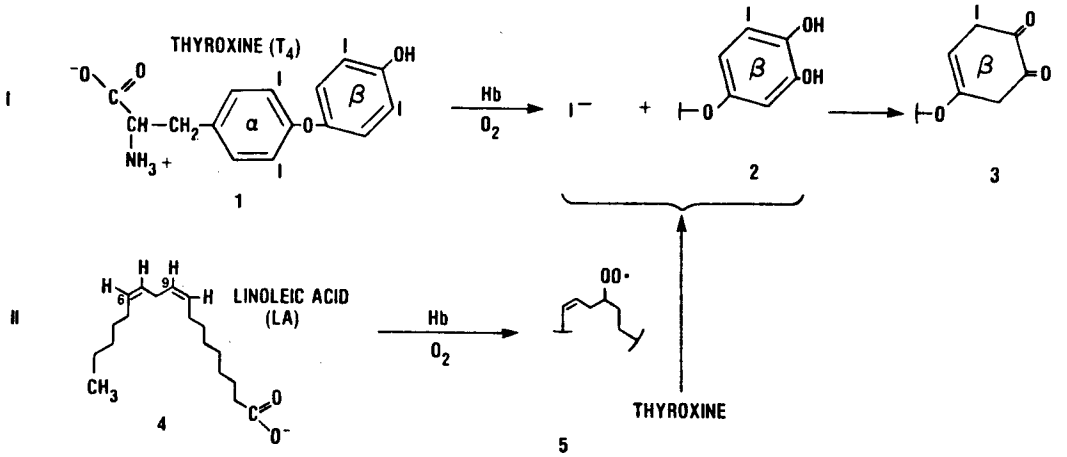

FIG. 3. Proposed mechanism for thyroxine deiodination and linoleic acid peroxidation by hemoglobin. Thyroxine can be oxidatively deiodinated in aqueous solutions containing hemoglobin to form the ortho-hydroxy derivative (compound 2), which can then oxidize to form the ortho-quinone (compound 3). Linoleic acid is also oxidized in the presence of hemoglobin and oxygen to form a free radical (compound 5) that can actively deiodinate thyroxine to yield compound 2.

TABLE 1

Relative Inhibition of Linoleic Acid Oxidation by Iodothyronines and Selected Known Antioxidants

Antioxidant	Concentration of antioxidant (molarity × 10^{-7})	Percent inhibition of linoleic acid peroxidation (ΔA234)[a]
0	--	0
RT_3	1.3	20.3 ± 3.4
	2.6	48.9 ± 1.8
T_4	1.3	15.7 ± 2.7
	2.6	41.0 ± 2.4
T_3	2.6	28.2 ± 1.7
3,5-T_2	2.6	39.1 ± 2.8
3,3'-T_2	2.6	25.0 ± 1.7
T_0	2.6	27.6 ± 0.7
α-Tocopherol	13.0	6.8 ± 2.8
	26.0	17.1 ± 3.4
Glutathione	13.0	0
	26.0	42.1 ± 2.2
Ascorbic acid	6.5	31.1 ± 5.1
	13.0	58.1 ± 1.6
DTT	13.0	49.1 ± 3.6

[a]Linoleic-acid peroxidation is measured as the difference in absorbance at 234 nm between 0 and 5 min of reaction (see Methods).

studies, uric acid has also been proposed as a physiologically important antioxidant (22). We estimate that in comparison to ascorbate, RT_3 is also more active than uric acid as an antioxidant.

Since the iodothyronines are fat-soluble, like α-tocopherol, their most likely physiologic role as antioxidants would probably be in the protection of membrane lipids. Wynn et al. previously reported that thyroxine is degraded during microsomal lipid peroxidation and suggested that the hormone plays an antioxidant role as a free-radical scavenger (23). In their assay, T_4 was more active than RT_3; vitamin E and ascorbic acid had no antioxidant effect at 10 μM. The discrepancy between this data and the present report may be because of the use of different fatty acid substrates as well as a different index (oxygen uptake) to assess the antioxidant effect.

Protection of Erythrocytes from Oxidative Lysis by Iodothyronines

Because earlier studies have shown that erythrocytes from vitamin E deficient mice are more subject to hemolysis than erythrocytes from euthyroid mice (20,24,25), we examined the ability of RT_3 to preserve red cell membrane integrity in vitro. The addition of 0.1 μM RT_3 to incubations containing erythrocytes and purified hemoglobin significantly decreased erythrocyte lysis in the presence of H_2O_2 (OD 525 nm, n = 5, 0.0695 ± 0.0021 with RT_3 compared with 0.0788 ± 0.001 without RT_3; differences significant at $p < 0.05$).

Physiologic Correlates

Iodothyronines may play an important antioxidant role in normal physiology. We have observed that 0.13 μM T_4 is effective in inhibiting the oxidation of linoleic acid (Table 1). This dose corresponds closely to the normal serum levels of T_4 (about 0.1 μM) in adult humans. The normal serum concentration of T_3 (about 2 nM), the most active hormone thermogenically, is not high enough to be effective as an antioxidant by our measures. Thus, the high levels of serum thyroxine (relative to T_3) produced by the thyroid gland may be acting both as a peripheral antioxidant and a prohormone (26) source for T_3.

If iodothyronines play a physiologic role as antioxidants, the hypothyroid state should result in oxidative damage to some components,

possibly membrane lipids. Hoch et al. have previously shown that hypothyroid rats, killed 6 hr after the injection of labeled linoleic acid, did not effectively convert linoleic acid to arachidonic acid relative to euthyroid animals (27). This decrease in 18:2 to 20:4 conversion could have resulted from an enhancement of alternative metabolic or degrative 18:2 pathways since the minor alterations in chain elongation or desaturase ($\Delta 6, \Delta 5$) activities in hypothyroidism do not account (28) for the decreased accumulation of arachidonic acid observed by Hoch et al. (27). Since our in vitro studies demonstrated an inhibition by iodothyronines of linoleic acid peroxidation, we conclude that euthyroid levels of iodothyronines may inhibit the degradation of linoleic acid in vivo by preserving unsaturated bonds.

An overall increase in the size of the red blood cells has been observed after thyroidectomy in animals, and in patients with untreated hypothyroidism, suggesting an alteration in membrane properties. The presence of small numbers of irregularly contracted red blood cells in blood smears from hypothyroid patients, also suggests an altered membrane structure (29).

Although similar studies have not been reported for the hyperthyroid case, this situation may be more complex as thyroid hormones are known to generally increase metabolic rate. Because free-radical production is a natural consequence of oxidative metabolism (30), hyperthyroidism may have a net effect of promoting lipid oxidation or at least be antagonistic to the potential antioxidant effects.

Although vitamin E is in the diet and is normally in relative molar abundance compared with iodothyronines, the blood levels of vitamin E are very low in certain physiologic instances. For example, low placental permeability of vitamin E (31) results in a low level of this vitamin in the human fetus; newborn babies have only about one-fifth of the adult level (32). However, during the prenatal period, RT_3 concentration is high in cord serum (315 ng/dl) and amniotic fluid (82 ng/dl) compared with normal adult serum (76 ng/dl) (33). In the adult, thyroxine is believed to be peripherally converted by deiodination to the more active hormone, T_3, while in the placenta and fetus T_4 is selectively converted to RT_3 through inner-ring deiodination (34,35). Although RT_3 has no previously identified physiologic function, a proposal has been made that in the adult, inner-ring deiodination may be a mechanism for thyroxine inactivation and iodine recovery (36). Because the present studies demonstrate that RT_3 is an effective inhibitor of linoleic-acid peroxidation and is also effective in preserving red cell membranes, we postulate that the high levels of RT_3 found in the developing fetus play an important antioxidant role during fetal development. Consistent with this view is the observation that serum RT_3 concentrations remain high during the first few days after delivery but are significantly lower by the fifth day and reach adult levels by the seventh day (37). Vitamin E levels reach normal adult levels by the sixth postnatal day in breast-fed babies (38).

T_3 is commonly accepted to act through a pathway mediated by nuclear receptors to modulate the expression of specific genes (39). We believe that the present data demonstrates an important alternative role for thyroid hormones as physiologic antioxidants.

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REFERENCES

1. Tseng, Y.L., and Latham, K.R. (1981) Abstract, 63rd Annual Meeting of the Endocrine Society.
2. Misra, H.P., and Fridovich, I. (1972) J. Biol. Chem. 247, 6960-6062.
3. Tappel, A.L. (1961) in Autoxidation and Antioxidants (Lundberg, W.O., ed.) Vol. 1, pp. 325-366, Interscience, New York.
4. Mengel, C.E. (1972) Ann. N.Y. Acad. Sci. 203, 163-171.
5. Wright, W.D. (1963) Nature 198, 1239-1244.
6. Bradford, M.M. (1976) Anal. Biochem. 72, 248-254.
7. Worthington Enzyme Manual (1972) pp. 25-26, Worthington Biochem. Corp., Freehold, NJ.
8. Salvati, S.M., Ambrogloni, M.T., and Tentori, L. (1969) Ital. J. Biochem. 18, 1-18.
9. Green, W.L. (1972) J. Chromatogr. 72, 83-91.
10. Svedberg, T., and Fahraeus, R. (1926) J. Am. Chem. Soc. 48, 430-438.
11. Marcial, R.M.B., Ozwa, Y., and Chopra, I.H. (1979) Endocrinology 104, 365-371.
12. Visser, T.J., van der Dors-Tobe, I., Docter, R., and Hennemann, G. (1976) Biochem. J. 157, 479-482.
13. Leonard, J.L., and Rosenberg, I.N. (1978) Endocrinology 103, 274-280.
14. Kessler, G., and Pileggi, V. (1968) J. Clin. Chem. 14, 811-815.
15. Smallridge, R.C., Burman, K.D., Ward, K.E., Wartofsky, L., Dimond, R.C., Wright, F.D., and Latham, K.R. (1981) Endocrinology 108, 2336-2345.
16. Lissitzky, P.S., and Bouchildoux, S. (1957) Bull. Soc. Chim. Biol. 39, 133-143.
17. Balsam, A., Sexton, F., Borges, M., and Ingbar, S.H. (1983) J. Clin. Invest. 72, 1234-1245.
18. Matsuura, T., Nagamachi, T., Nishinaga, H.K., and Cahnmann, H.J. (1969) J. Org. Chem. 34, 2554-2558.

19. Plaskett, L.G. (1961) Biochem. J. 78, 652-657.
20. Grimes, A.J. (1980) in Human Red Cell Metabolism, pp. 14-15, Blackwell Scientific Publications, Oxford, England.
21. Tappel, A.L. (1973) Fed. Proc. 32, 1870-1874.
22. Ames, B.N., Cathcart, R., Schwiers, E., and Hochstein, P. (1981) Proc. Natl. Acad. Sci. USA 78, 6858-6862.
23. Wynn, J. (1968) Arch. Biochem. Biophys. 126, 880-891.
24. Rose, S., and György, P. (1952) Am. J. Physiol. 168, 414-420.
25. McCay, P.B., Poyer, J.L., Pfeifer, P.M., May, H.E., and Gilliam, J.M. (1971) Lipids 6, 297-306.
26. Oppenheimer, J.H., Schartz, H.L., and Surks, M.I. (1972) J. Clin. Invest. 51, 2493-2497.
27. Hoch, F.L., Subramanian, C., Phopeshwarker, G.A., and Mead, J.F. (1981) Lipids 16, 328-335.
28. de Gomez Dumm, I.N.T., de Alaniz, M.J.T., and Brenner, R.R. (1977) Adv. Exp. Med. Biol. 83, 609-616.
29. Horton, L., Coburn, R.J., England, J.M., and Himsworth, R.L. (1976) Q. J. Med. (NS) 45, 101-124.
30. Harman, D. (1972) Am. J. Clin. Nutr. 25, 839-843.
31. György, P., Cogan, G., and Rose, C. (1952) Proc. Soc. Exp. Biol. Med. 81, 536-538.
32. Wright, S.W. (1951) Paediatrics 7, 386-393.
33. Burman, K.D., Read, J., Dimond, R.C., Strum, O., Wright, F.D., Patow, W., Earll, J.M., and Wartofsky, L. (1976) J. Clin. Endocrinol. Metab. 43, 1351-1359.
34. Roti, E., Braverman, L.E., Fang, S.L., Alex, S., and Emerson, C.H. (1982) Endocrinology 111, 959-963.
35. Banovac, K., Bzik, L., Tislaric, T., and Sekso, M. (1980) Hormone Res. 12, 253-259.
36. Burman, K.D. (1978) Metabolism 27, 615-630.
37. Chopra, I.J., Sack, J., and Fisher, D.A. (1975) J. Clin. Invest. 55, 1137-1144.
38. Nakano, M., Tsutsumi, Y., and Ushijima, Y. (1971) Biochim. Biophys. Acta 252, 335-347.
39. Latham, K.R., MacLeod, K.M., Papavasiliou, S.S., Martial, J.A., Seeburg, P.H., Goodman, H.M., and Baxter, J.D. (1978) in Receptors and Hormone Action (O'Malley, B.W., and Birnbaumer, L., eds.) Vol. 3, pp. 76-100, Academic Press, New York.

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Pathways of Acetyl CoA Production for Lipogenesis from Acetoacetate, β-Hydroxybutyrate, Pyruvate and Glucose in Neonatal Rat Lung

PATRICIA M. SHEEHAN and **YU-YAN YEH,*** *Laboratories of Nutrition and Metabolism, St. Jude Children's Research Hospital, Memphis, TN 38101 and Department of Physiology and Biophysics, University of Tennessee Center for the Health Sciences, Memphis, TN 38163*

ABSTRACT

The rate of fatty acid synthesis from acetoacetate (AcAc) is 2-3 times greater than from glucose in developing rat lung. To determine the reason for this difference, we investigated the pathways of lipogenesis from [3-^{14}C] AcAc, [3-^{14}C] β-hydroxybutyrate (βOHB), [U-^{14}C] glucose or [2-^{14}C] pyruvate in minced lung tissue of 3- to 4-day-old rats. The addition of (−)hydroxycitrate, an inhibitor of ATP-citrate lyase, inhibited fatty acid synthesis from glucose, pyruvate, and βOHB by 88%, 70% and 60%, respectively, but had no effect on that from AcAc. Benzene 1,2,3-tricarboxylate, an inhibitor of tricarboxylate translocase, inhibited fatty acid synthesis from all substrates by at least 50%. Incubation with aminooxyacetate, an inhibitor of aspartate aminotransferase, had no effect on lipid synthesis from glucose, pyruvate or AcAc, but increased lipid synthesis from βOHB. Results indicate that for lipid synthesis in the neonatal lung, acetyl CoA from AcAc is derived predominantly from a cytoplasmic pathway involving AcAcCoA synthetase and AcAcCoA thiolase, whereas citrate is the major route of acetyl group transfer from glucose. Lipogenesis from βOHB involves both the cytoplasmic and citrate pathways.

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INTRODUCTION

In the lung, de novo fatty acid synthesis is important because of its contribution to the production of pulmonary surfactant, the lipid-protein complex that maintains alveolar stability (1-4). The source of acetyl CoA for lipid synthesis in the neonatal lung, however, has been unclear. Ketone bodies have been established as important lipid precursors in the neonatal rat brain (5-7), and they are metabolized more rapidly than glucose. This rapid incorporation has been explained by a cytoplasmic pathway of sterol and fatty acid synthesis (5,8, 9). Indeed, the production of acetyl CoA via the cytoplasmic acetoacetyl CoA (AcAcCoA) synthetase and thiolase pathway appears to predominate in the developing rat brain (5,9, 10).

In a study using $^{3}H_2O$, Todhunter and Scholz (11) observed a marked inhibition of de novo pulmonary fatty acid synthesis in the presence of (−)hydroxycitrate in adult rat lungs, indicating that the ATP-citrate lyase pathway is the major route of acetyl CoA generation in the adult lung, regardless of substrate. Similar results were reported in an in vitro study with glucose as the substrate (12). Recently, however, we demonstrated the importance of ketone bodies as lipid precursors in the developing rat lung (13,14). We also observed a more rapid synthesis of lipids from acetoacetate (AcAc) than from glucose (14) and showed that the two cytoplasmic enzymes, AcAcCoA synthetase and AcAcCoA thiolase, required for converting ketone bodies to acetyl CoA, are present in the developing rat lung (13). Furthermore, the developmental pattern of these enzymes parallels that of AcAc incorporation into fatty acids; the accelerated rate of lipogenesis during the early postnatal period coincides with increased activities of these enzymes during the same period (13,14). The cytoplasmic pathway of fatty acid synthesis from ketone bodies, therefore, may predominate in the neonatal lung and, as in the brain, be responsible for the more rapid use of AcAc compared with glucose. To test this hypothesis, we measured rates of lipid synthesis from AcAc, β-hydroxybutyrate (βOHB), glucose and pyruvate in the presence or absence of specific inhibitors of the citrate pathway.

MATERIALS AND METHODS

Animals

Sprague-Dawley rats were raised by breeding procedures described previously (15). For this study, we used rats that were 3 to 4 days old because lung lipid synthesis is elevated in rats of this age compared with older suckling rats (14).

*To whom correspondence should be addressed.

Metabolic Studies

The rats were killed and lung tissues removed, dissected free of vascular tissue and minced for metabolic experiments as described previously (14). Before mincing, the tissues were not perfused to remove residual blood because perfusion with an isotonic solution increased water retention and tissue weight. Nevertheless, tissues with apparent blood contamination were not used. The incubation system, in a final volume of 2 ml, consisted of Ca^{2+}-free Krebs-Ringer bicarbonate buffer, pH 7.4; 33 mg bovine serum albumin; and 20 μmol of [3-^{14}C]AcAc, [2-^{14}C]pyruvate or [U-^{14}C]glucose, or 10 μmol of [3-^{14}C]D-βOHB containing 1.0 μCi radioactivity. When the incubation system included ketone bodies, it also contained 20 μmol of glucose. Substrate concentrations were chosen because, in preliminary experiments, lipid synthesis from substrates was linear from 5 to 20 mM (14). Other additions, as indicated in each table, included (−)hydroxycitrate (1 or 5 mM), benzene 1,2,3-tricarboxylate (5 mM), or aminooxyacetate (1 mM). In preliminary experiments, we tried several concentrations of each inhibitor; the concentrations used in these experiments were the lowest to yield maximum inhibition of lipid synthesis. The details of the incubation have been described (14).

After incubation, the tissues were separated from the buffer by centrifugation (1,500 × g, 10 min), washed twice in cold 0.9% saline and homogenized in 2 ml of distilled water. From each sample, 1 ml aliquots were transferred to tubes containing 20 ml of chloroform/methanol (2:1, v/v) for lipid extraction (16), then the samples were dried under nitrogen.

The extracted lipids were resuspended in 500 μl of the chloroform/methanol mixture, and an aliquot of 50 μl was used to determine the radioactivity in the total lipids. The remaining lipid suspension was saponified with 3 ml of 3.75% KOH in methanol for 4 hr at 70 C. Fatty acids and nonsaponifiable lipids (cholesterol and other sterols) were extracted with petroleum ether (bp 50-60) and assessed for radioactivity as previously described (6). Glyceride-glycerol synthesized from each substrate was determined by the method of Seccombe et al. (17) used in the previous studies (13,14), except that a blank was also carried through the saponification procedure. We found the blank was necessary to correct for radioactivity nonspecific to glyceride-glycerol present in the aqueous phase.

The radioactivity of CO_2 and lipid fractions was determined by liquid scintillation counting (Model Mark III, Searle Analytic, Inc., Des Plaines, IL). Lipid samples were counted in a toluene scintillation fluid (4 g Omnifluor, 230 ml ethanol and 770 ml toluene) and glycerol samples were counted in ACS (Aqueous Counting Scintillant).

Chemicals

[U-^{14}C]Glucose, [2-^{14}C]pyruvate, ethyl[3-^{14}C]AcAc, [3-^{14}C]D-βOHB and Omnifluor were purchased from New England Nuclear, Boston, MA. Ethyl[3-^{14}C]AcAc was converted to [3-^{14}C]AcAc by the method of Krebs and Eggleston (18). ACS was purchased from Amersham Corp., Arlington Heights, IL. Benzene 1,2,3-tricarboxylate and aminooxyacetic acid were from K & K Laboratories, Plainview, NY, and (−)hydroxycitrate was a gift from Dr. D. Ingle, American Cyanamid Co., Princeton, NJ. Organic solvents for lipid extraction were purchased from Fisher Scientific Co., Pittsburgh, PA. Bovine serum albumin (Fraction V), substrates and other chemicals were purchased from Sigma Chemical Co., St. Louis, MO.

Statistical Analysis

Mean values were compared using Student's *t*-test.

RESULTS

In the presence of (−)hydroxycitrate, a competitive inhibitor of ATP-citrate lyase (19), fatty acid synthesis from glucose and pyruvate was decreased by 88% and 70% from control values, respectively (Table 1). The incorporation of glucose and pyruvate into total lipid was inhibited by 47% and 58%, respectively. In contrast, fatty acid synthesis from AcAc in the presence of (−)hydroxycitrate was comparable to the control value. Synthesis of fatty acid from βOHB, however, was reduced by 60%. Total lipid synthesis from AcAc and βOHB followed a similar trend: (−)hydroxycitrate caused no significant decrease in lipid synthesis from AcAc, but caused a 61% decrease in synthesis from βOHB. (−)Hydroxycitrate had no effect on energy production from AcAc or βOHB, but inhibited CO_2 production from pyruvate by 15% and from glucose by 32%. In contrast to the previous studies (13,14), no significant synthesis of glyceride-glycerol from the ketone bodies under the experimental conditions was observed. This discrepancy may be attributed to the analytic procedure described in *Materials and Methods.* Glyceride-glycerol synthesis from glucose was decreased 17% by (−)hydroxycitrate. No change in glyceride-glycerol synthesis from pyruvate occurred in the presence of (−)hydroxycitrate. Unlike the studies with glucose, AcAc and βOHB, the

TABLE 1

Effect of (−)Hydroxycitrate on Incorporation of ^{14}C-Labeled Substrates into CO_2 and Lipids in Lungs of 3- to 4-Day-Old Rats

Substrate	(−)Hydroxycitrate concentration (mM)	CO_2	Total lipids	Fatty acids	Glyceride-glycerol
		(nmol substrate incorporated/g tissue per 2 hr)[a]			
[3-^{14}C] Acetoacetate	0	9595 ± 450	857 ± 77	658 ± 51	–
	1	9385 ± 264	754 ± 65	594 ± 44	–
[3-^{14}C] D-β-Hydroxybutyrate	0	2182 ± 93	283 ± 12	203 ± 7	–
	1	2256 ± 78	110 ± 9**	82 ± 6**	–
[U-^{14}C] Glucose	0	3344 ± 182	603 ± 17	243 ± 16	317 ± 9
	1	2293 ± 71**	320 ± 12**	28 ± 2**	263 ± 4**
[2-^{14}C] Pyruvate	0	15842 ± 991	937 ± 102	607 ± 65	110 ± 10
	5	13435 ± 122*	398 ± 9**	181 ± 4**	99 ± 10

[a]Values are means ± SEM for 5 samples. A value that is significantly different from that in the absence of (−)hydroxycitrate is indicated as: *($P < 0.05$); **($P < 0.01$).

maximal inhibition of pyruvate use required 5 mM instead of 1 mM of (−)hydroxycitrate.

Benzene 1,2,3-tricarboxylate is an inhibitor of the mitochondrial tricarboxylate translocase (20). When tissues were incubated with this inhibitor, the rates of energy production, total lipid synthesis, fatty acid synthesis and glyceride-glycerol synthesis from glucose were decreased by 40%, 50%, 64%, and 39%, respectively (Table 2). This compound also suppressed fatty acid synthesis from pyruvate, βOHB and AcAc by 53%, 48% and 50%, respectively. While the rates of total lipid synthesis from these three substrates were significantly depressed (32-47%), their conversion to CO_2 and glyceride-glycerol was not affected by this inhibitor. Nonsaponifiable lipid synthesis from glucose and pyruvate was inhibited to a lesser extent than fatty acid synthesis: 43% and 28% respectively, whereas that from βOHB and AcAc was not inhibited.

The tissues were incubated in the presence of aminooxyacetate to investigate the possible contribution of N-acetylaspartate as an intermediate for the transfer of mitochondrial acetyl units for fatty acid synthesis in neonatal rat lung (21). Aminooxyacetate blocks synthesis of N-acetylaspartate by preventing its intramitochondrial production from glutamate (22). This inhibitor did not alter total lipid or fatty acid synthesis from any substrate except βOHB (Table 3). In contrast to the inhibitory effects of (−)hydroxycitrate and benzene 1,2,3-tricarboxylate, aminooxyacetate stimulated the rate of lipogenesis from βOHB and the rate of CO_2 production from ketone bodies and pyruvate.

Combining (−)hydroxycitrate and aminooxyacetate resulted in the inhibition of total lipid and fatty acid synthesis, as with (−)hydroxycitrate alone, and increased CO_2 production from ketones, as with aminooxyacetate alone (data not shown).

DISCUSSION

Lipogenesis, as an extramitochondrial process, depends on the availability of acetyl CoA in the cytoplasm (23,24). Acetyl CoA, in turn, is produced via mitochondrial and cytoplasmic pathways. The incorporation of glucose into fatty acids, for example, requires intramitochondrial synthesis of acetyl CoA, its conversion to an intermediate such as citrate, acetate, acetylcarnitine or N-acetylaspartate, transport of the intermediate across the mitochondrial membrane, and the regeneration of acetyl CoA in the cytoplasm (9,12,23,25). In rat brain and liver, the majority of acetyl CoA generated from glucose is translocated as citrate, which is cleaved by ATP-citrate lyase to form cytoplasmic acetyl CoA (8,9,25). N-Acetylaspartate also contributes to acetyl CoA transfer in the brain (9,26,27).

In contrast to glucose, the formation of acetyl CoA from AcAc does not necessarily involve the mitochondria (5,9). AcAc can be converted to acetyl CoA in the cytosol by AcAc-CoA synthetase and AcAcCoA thiolase (28). Alternatively, acetyl CoA can be generated from AcAc by 3-oxoacid CoA transferase and AcAcCoA thiolase in the mitochondria, transported to the cytoplasm via citrate and regenerated by the same pathway as acetyl CoA from glucose. Similarly, after its dehydrogenation to AcAc in the mitochondria, βOHB can be incorporated into lipids through the formation of

TABLE 2

Effect of Benzene 1,2,3-Tricarboxylate on Incorporation of ^{14}C-Labeled Substrates into CO_2 and Lipids in Lungs of 3- to 4-Day-Old Rats

Substrate	Benzene 1,2,3-tricarboxylate (mM)	CO_2	Total lipids	Fatty acids	Nonsaponifiable lipid	Glyceride-glycerol
		(nmol substrate incorporated/g tissue per 2 hr)[a]				
[3-^{14}C] Acetoacetate	0	8831 ± 488	728 ± 89	515 ± 58	71 ± 10	—
	5	9084 ± 167	389 ± 20**	259 ± 13**	64 ± 6	—
[3-^{14}C] D-β-Hydroxybutyrate	0	1992 ± 121	254 ± 33	184 ± 24	19 ± 4	—
	5	2276 ± 69	147 ± 11*	95 ± 9**	16 ± 1	—
[U-^{14}C] Glucose	0	3270 ± 106	606 ± 39	211 ± 22	30 ± 2	274 ± 18
	5	1959 ± 45**	300 ± 26**	77 ± 6**	17 ± 1*	166 ± 15**
[2-^{14}C] Pyruvate	0	15842 ± 991	937 ± 102	607 ± 65	154 ± 18	110 ±10
	5	14557 ± 759	636 ± 48*	283 ± 25**	111 ± 6*	153 ±16

[a] Values are means ± SEM for 5 samples. A value that is significantly different from that in the absence of benzene 1,2,3-tricarboxylate is indicated as: *($P < 0.05$); **($P < 0.01$).

acetyl CoA by either of these pathways. In the present experiments, we investigated the importance of the cytoplasmic pathway for acetyl CoA production from 4 potentially important substrates for the neonatal rat lung: AcAc, βOHB, pyruvate and glucose.

Agreeing with other observations on adult rat lung (11,12), our results demonstrate that in neonatal rat lung, the ATP-citrate lyase pathway is the major route of acetyl group transfer for lipogenesis from glucose and pyruvate. In contrast, acetyl CoA synthesis from AcAc occurs almost exclusively by the cytoplasmic pathway in neonatal rat lung, as indicated by the lack of effect of (−)hydroxycitrate on fatty acid synthesis (Table 1). However, benzene 1,2,3-tricarboxylate inhibited the incorporation of AcAc into fatty acids by 50%. This depression in lipogenesis may be attributable to the inhibitory effect of benzene 1,2,3-tricarboxylate on acetyl CoA carboxylase and the consequent reduction in malonyl CoA production (8). The inhibitor had no significant effect on nonsaponifiable lipid synthesis from AcAc. The nonsaponifiable fraction consists primarily of cholesterol, which requires acetyl CoA but not malonyl CoA for its synthesis. Therefore, if the inhibition of fatty acid synthesis was from a lack of citrate translocation, nonsaponifiable lipid synthesis would have been inhibited to a similar extent. For lipid synthesis from βOHB, our data indicate that the ATP-citrate lyase pathway and the cytoplasmic AcAcCoA synthetase and thiolase pathway are equally important. N-Acetylaspartate does not appear to play a role in acetyl transfer from ketone bodies, glucose or pyruvate in neonatal rat lung, as has been demonstrated in adult rat lung (12).

These observations provide an explanation for the more rapid incorporation of AcAc into fatty acids compared with glucose in neonatal rat lung (14). Acetyl CoA generated in the cytoplasm is immediately available for lipogenesis, whereas that produced in mitochondria must be converted to an intermediate (such as citrate), transported across the mitochondrial membrane, and regenerated in the cytoplasm. Further support for the importance of the cytoplasmic pathway of acetyl CoA production from AcAc in neonatal rat lung is provided by our previous observation that the activities of cytoplasmic AcAcCoA synthetase and AcAc-CoA thiolase increase along with the rates of fatty acid synthesis during the early suckling period (13).

We observed increases in CO_2 production from ketone bodies and pyruvate, and fatty acid synthesis from βOHB in the presence of aminooxyacetate. The mechanism(s) underlying

TABLE 3

Effect of Aminooxyacetate on Incorporation of ^{14}C-Labeled Substrates into CO_2 and Lipids in Lungs of 3- to 4-Day-Old Rats

Substrate	Aminooxyacetate (mM)	CO_2	Total lipids	Fatty acids	Glyceride-glycerol
		(nmol substrate incorporated/g tissue per 2 hr)[a]			
[3-^{14}C] Acetoacetate	0	8056 ± 415	597 ± 46	455 ± 31	–
	1	11660 ± 1060*	656 ± 69	507 ± 59	–
[3-^{14}C] D-β-Hydroxybutyrate	0	1559 ± 85	193 ± 16	142 ± 9	–
	1	2632 ± 193**	261 ± 21*	201 ± 19*	–
[U-^{14}C] Glucose	0	2872 ± 250	542 ± 38	205 ± 22	274 ± 33
	1	3113 ± 179	450 ± 28	156 ± 12	233 ± 10
[2-^{14}C] Pyruvate	0	15842 ± 991	937 ± 102	607 ± 65	110 ± 10
	1	19157 ± 342*	1965 ± 49	640 ± 60	101 ± 11

[a]Values are means ± SEM for 5 samples. A value that is significantly different from that in the absence of aminooxyacetate is indicated as: *($P < 0.05$); **($P < 0.01$).

the stimulation could not be identified from the present data. Aminooxyacetate is an inhibitor of pyridoxal phosphate requiring enzymes (22). The inclusion of this compound in the incubations would be expected to alter several metabolic pathways in addition to the synthesis of N-acetylaspartate. Whether such nonspecific actions lead to the changes in energy and lipid production remain to be determined.

The reason for the depression of CO_2 production from glucose by (−)hydroxycitrate is not apparent. Research has suggested that (−)hydroxycitrate inhibits glycolysis, possibly at phosphofructokinase, but not the Krebs cycle (29). This possibility seems reasonable since the oxidation of pyruvate was inhibited to a lesser degree than that of glucose, and may also account for the greater inhibition of fatty acid synthesis from glucose compared with pyruvate and for the inhibition of glyceride-glycerol synthesis from glucose but not pyruvate in the presence of (−)hydroxycitrate.

In neonatal rats, plasma concentrations of ketone bodies increase from 0.2-0.3 mM at birth to 1-2 mM during the first week of life (30,31). During the same period, glucose concentrations range from 4-7 mM. However, higher concentrations (e.g. βOHB, 5 mM; AcAc, glucose, and pyruvate, 10 mM) were used in the inhibitory studies because these concentrations had been established previously as yielding maximal rates of lung lipid synthesis (14). Although we have previously reported that pulmonary fatty acids are synthesized from ketone bodies and glucose in vivo (13), care should be taken in directly extrapolating the present results to in vivo conditions.

Consistent with our earlier studies (13,14) demonstrating the importance of ketone bodies as precursors of lung lipid, the rates of AcAc incorporation into lipids in vitro (Tables 1-3) were consistently higher than those of glucose. The study also suggests the potential contribution of pyruvate to lung lipids because of its greater rate of use than glucose for fatty acid synthesis.

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REFERENCES

1. Buechler, K.F., and Rhoades, R.A. (1981) Biochim. Biophys. Acta 665, 393-398.
2. Jobe, A. (1979) Biochim. Biophys. Acta 572, 404-412.
3. Van Golde, L.M.G. (1976) Am. Rev. Resp. Dis. 114, 977-1000.
4. Perelman, R.H., Engle, M.J., and Farrell, P.M. (1981) Lung 159, 53-80.
5. Yeh, Y.-Y., Streuli, V.L., and Zee, P. (1977) Lipids 12, 957-964.
6. Yeh, Y.-Y. (1980) Lipids 15, 904-907.
7. Webber, R.J., and Edmond, J. (1977) J. Biol. Chem. 252, 5222-5226.
8. Patel, M.S., and Owen, O.E. (1976) Biochem. J. 156, 603-607.
9. Patel, T.B., and Clark, J.B. (1980) Biochem. J. 188, 163-168.
10. Webber, R.J., and Edmond, J. (1979) J. Biol. Chem. 254, 3912-3920.
11. Todhunter, D.A., and Scholz, R.W. (1980) Am. J. Physiol. 239, E407-E411.
12. Evans, R.M., and Scholz, R.W. (1977) Am. J. Physiol. 232, E358-E363.
13. Yeh, Y.-Y. (1982) Int. J. Biochem. 14, 81-86.

14. Sheehan, P.M., and Yeh, Y.-Y. (1983) Lipids 18, 295-301.
15. Yeh, Y.-Y., Streuli, V.L., and Zee, P. (1977) Lipids 12, 367-374.
16. Folch, J., Lees, M., and Sloan-Stanley, G.H. (1957) J. Biol. Chem. 226, 497-509.
17. Seccombe, D.W., Harding, P.G.R., and Possmayer, F. (1977) Biochim. Biophys. Acta 488, 402-416.
18. Krebs, H.A., and Eggleston, L.V. (1945) Biochem. J. 39, 408-419.
19. Watson, J.A., Fang, M., and Lowenstein, J.M. (1969) Arch. Biochem. Biophys. 135, 209-217.
20. Robinson, B.R., Williams, G.R., Halperin, M.L., and Leznoff, C.C. (1971) Eur. J. Biochem. 20, 65-71.
21. Rous, S. (1973) Life Sci. 13, 1715-1724.
22. Hopper, S., and Segal, H.L. (1964) Arch. Biochem. Biophys. 105, 501-505.
23. Brady, R.O., Bradley, R.M., and Trams, E.G. (1960) J. Biol. Chem. 235, 3093-3098.
24. Lowenstein, J.M. (1968) in Metabolic Roles of Citrate (Goodwin, T.W., ed.) pp. 61-86, Academic Press, London and New York.
25. Watson, J.A., and Lowenstein, J.M. (1970) J. Biol. Chem. 245, 5993-6002.
26. D'Adamo, A.F. Jr., and Yatsu, F.M. (1966) J. Neurochem. 13, 961-965.
27. D'Adamo, A.F. Jr., Gidez, L.I., and Yatsu, F.M. (1968) Exp. Brain Res. 5, 267-273.
28. Buckley, B.M., and Williamson, D.H. (1973) Biochem. J. 132, 653-656.
29. Lowenstein, J.M. (1971) J. Biol. Chem. 246, 629-632.
30. Page, M.A., Krebs, H.A., and Williams, D.H. (1971) Biochem. J. 121, 49-53.
31. Yeh, Y.-Y., and Zee, P. (1976) Pediatr. Res. 10, 192-197.

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Effects of Different Levels of Dietary *trans*-Octadecenoate on Steroid Metabolism in Rats[1]

MIDORI WATANABE, YOUNG-J. CHO, TAKASHI IDE and MICHIHIRO SUGANO*,
Laboratory of Nutrition Chemistry, Kyushu University School of Agriculture 46-09, Higashi-ku, Fukuoka 812, Japan

ABSTRACT

Rats were fed semipurified diets containing olive oil or partially hydrogenated corn oil at the 5 or 20% level for ca. 30 days. These fat diets contained the same amount of octadecenoate but differed in the geometry with respect to each fat level. Contents of *t*-18:1 were 26% and 41% of total fatty acids, respectively. The linoleic acid content was also made equivalent (3.8 energy %). After feeding on cholesterol-free diets, rats on *trans* fat, compared to those on *cis* fat, showed: (a) no changes in serum cholesterol and apolipoprotein levels, (b) no effects on the bile flow and concentrations of biliary cholesterol or bile acids, (c) a trend toward increased fecal excretion of neutral and acidic steroids, (d) a lesser extent of transformation of cholesterol to coprostanol in the gut, and (e) no changes in the composition of biliary and fecal bile acids. Observations (c) and (d) were more marked with a high *trans* fat regimen. These observations, except for serum apolipoproteins and fecal steroid excretion, were practically reproducible even when rats were fed cholesterol-enriched diets.
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INTRODUCTION

Most studies involved in biological implications of dietary *trans* fats have been exclusively focused on effects, either on plasma cholesterol and atherosclerosis, or on the metabolism of *cis* fatty acids (1-3). Although the epidemiological investigation of Enig et al. (4,5), on the correlation between dietary *trans* fat and mortality from several cancers, has become the focus of criticisms (6-8), it seems important to confirm empirically whether dietary *trans* fat has such an effect, since both types and amounts of dietary fat have been implicated as causal factors in the incidence of certain types of cancer (9,10). Awad (11,12) recently observed a possible growth-promoting effect of elaidic acid compared to olive oil in Erlich tumor cells in mice, although there was criticism of these studies (13).

The increase in intraluminal cholesterol (14) and/or bile acids (15) is reported to be responsible for colon carcinogenesis; dietary fat crucially determines the amounts of fecal steroids (16,17). In a series of studies, we observed an increase in fecal steroid excretion in rats fed *trans*-octadecenoate compared to the *cis* counterpart (18). In the present paper, to determine the threshold level of dietary *trans* fat necessary to enhance fecal steroid excretion, the effects of different dietary levels of *trans*-octadecenoate on biliary and fecal steroid excretion were compared to those of *cis*-octadecenoate. The concentrations of serum lipids and apolipoproteins were also determined.

MATERIALS AND METHODS

Animals and Diets

Specific pathogen-free male Sprague-Dawley rats, purchased from the local breeder (Seiwa Experimental Animals, Ltd., Fukuoka), and weigh-ca. 100 g, were housed individually in a temperature-controlled room (20-23 C) with illumination from 0800 to 2000 hr. They were divided into 4 groups of 8 or 9 in each experiment with cholesterol-free or cholesterol-enriched (0.5%) diets. Each group was fed, ad libitum, a semipurified diet (Table 1) containing 2 different levels (5 and 20%) of olive oil and partially hydrogenated corn oil (18). The linoleic acid contents of paired *cis* and *trans* fat diets were adjusted to be equivalent by replacing a portion of these fats with safflower oil. Thus, all diets supplied linoleic acid at 3.8% of energy. After 28-29 days on the diets, ca. 0.3 ml of blood was withdrawn from the tail vein for cholesterol and apolipoprotein assay (18,19). During the days 30-35, the bile duct was cannulated under diethyl ether anesthesia at 1000-1100 hr. Rats were kept in restraining cages and the bile collected for 2 hr; the flow rate was measured at 30-min intervals. The bile was kept frozen until analyzed. The bile, drained at constant rate, was used for steroid analysis. After the bile drainage, rats were killed by decapitation, blood was collected and the liver and epididymal adipose tissue excised. Feces were collected for 2 days during days 26-28 and lyophilized.

Analyses

Liver lipids, biliary and fecal steroids and fatty acid compositions of tissue lipids (post-drainage serum, liver and adipose tissue) were analyzed as described in the preceding papers (18,19). Differences between group means were evaluated by two-way analysis of variance and subsequent t-test between the same fat level and between different levels of the same fat (20).

[1]A preliminary part of this study was presented at the 74th annual AOCS meeting, Chicago, 1983.

*To whom correspondence should be addressed.

TABLE 1

Composition of Diets

Ingredients	5% Fat		20% Fat	
	OL	PHC	OL	PHC
	(%)		(%)	
Casein	20	20	20	20
Olive oil (OL)	2.9	-	18.4	-
Partially hydrogenated corn oil (PHC)	-	2.7	-	17.3
Safflower oil	2.0	2.2	1.5	2.6
Mineral mixture[a]	4	4	4	4
Vitamin mixture				
Water-soluble[a]	1	1	1	1
Fat-soluble[b]	0.1	0.1	0.1	0.1
Choline chloride	0.15	0.15	0.15	0.15
Cellulose powder	2	2	2	2
Sucrose[c]	67.85	67.85	52.85	52.85
Fatty acid composition (%)				
16:0	8.9	10.6	10.4	13.0
18:0	2.9	5.7	3.3	7.8
t-18:1	-	26.1	-	41.4
c-18:1	51.4	22.0	73.7	26.9
cc-18:2	35.3	35.2	10.5	10.5
ccc-18:3	0.2	0.2	0.4	0.1

[a]Harper mixture purchased from Oriental Yeast Co., Tokyo.
[b]Fat-soluble vitamins (retinyl palmitate 400 IU, cholecalciferol 200 IU and DL-α-tocopheryl acetate 10 mg per 100 g diet) dissolved in corn oil.
[c]Cholesterol, 0.5%, was added at the expense of sucrose.

RESULTS

Growth Parameters and Liver Weight

When cholesterol-free diets were fed, both food intake and weight gain were reduced moderately in rats fed high fat, compared to low fat, diets. Weight gain was slightly greater on *cis* fat than on *trans* fat (Table 2). Similar response patterns were observed when diets rich in cholesterol were fed. Relative liver weights were comparable in each group for cholesterol-free and cholesterol-enriched diets.

Serum and Liver Lipids

Serum and liver lipid data are presented in Table 2. When diets free of cholesterol were fed, serum cholesterol levels were comparable among groups, except for the high *cis* fat group in which cholesterol values were clearly elevated. Concentrations of liver cholesterol and triglyceride also responded similarly. The addition of cholesterol to diets increased the serum cholesterol levels of all groups of rats to a similar extent and no dietary fat-dependent differences were observed. However, concentrations of liver cholesterol and triglyceride were considerably higher in rats fed a 20% *cis* fat diet than in any other group.

On feeding cholesterol-free diets, there were no significant changes in serum apolipoproteins, although apo A-I tended to be lower on 5% *trans* fat than on 5% *cis* fat. In rats fed cholesterol-enriched diets, serum apo B was elevated significantly on feeding high fat diets, particularly the 20% *cis* fat diet.

Biliary Steroid Excretion

The biliary steroid data are presented in Table 3. In rats fed cholesterol-free diets, the bile flow rates were comparable in all groups. The increase in dietary *cis* fat, but not *trans* fat, elevated the biliary cholesterol levels significantly. No differences were noticed in concentrations and compositions of biliary bile acids. The bile flow rates and biliary bile acid levels were also comparable in rats fed cholesterol-enriched diets, but dietary cholesterol tended to increase these parameters, whereas cholesterol values remained unaffected. Varying the type and amount of dietary fats had no consistent effects on biliary steroid levels and compositions.

Fecal Steroid Excretion

Table 4 presents the results of fecal steroid excretion in rats fed diets free of cholesterol. The stool weights were comparable in each group. Daily neutral steroid excretion was enhanced on high fat diets, compared to low fat diets, and on a high *trans* fat diet, compared to the corresponding *cis* fat diet. The microbial transformation of choles-

TABLE 2

Effects of Dietary Fats on Serum and Liver Lipids

Lipids	5% Fat		20% Fat	
	OL	PHC	OL	PHC
Cholesterol-free diets				
Weight gain (g)[a]	191 ± 5[b]	176 ± 10	183 ± 8	171 ± 8
Food intake (g/day)	16.8 ± 0.3	16.3 ± 0.5	14.7 ± 0.4	14.0 ± 0.4
Serum				
Cholesterol (mg/dl)	74.0 ± 6.0	80.2 ± 3.8	110 ± 14[d]	89.6 ± 6.1
Apolipoproteins (mg/dl)				
Apo A-I	101 ± 8	78.1 ± 4.2	88.9 ± 1.5	94.6 ± 6.8
Apo B	2.2 ± 0.7	2.9 ± 0.6	3.0 ± 0.7	3.1 ± 0.5
Apo E	33.1 ± 1.4	33.3 ± 2.4	24.6 ± 0.7	30.6 ± 3.1
Liver				
Weight (g/100g body weight)	3.99 ± 0.21	3.80 ± 0.17	3.72 ± 0.11	3.74 ± 0.11
Cholesterol (mg/g)[c]	2.51 ± 0.07	2.78 ± 0.10	7.07 ± 0.74[de]	2.61 ± 0.19
Triglyceride (mg/g)	19.0 ± 0.7	18.9 ± 2.2	39.3 ± 2.7[de]	17.1 ± 3.1
Cholesterol-enriched diets				
Weight gain (g)[a]	227 ± 4	224 ± 3[e]	189 ± 6[d]	209 ± 5
Food intake (g/day)	19.2 ± 0.9	18.8 ± 0.6[e]	14.2 ± 0.4[d]	14.7 ± 0.6
Serum				
Cholesterol (mg/dl)	168 ± 12	145 ± 10	158 ± 8	141 ± 14
Apolipoproteins(mg/dl)				
Apo A-I	83.6 ± 2.6	86.4 ± 2.1	84.2 ± 2.7	87.8 ± 2.9
Apo B	2.9 ± 0.2	2.8 ± 0.2[e]	6.1 ± 7.8[de]	4.2 ± 0.4
Apo E	23.2 ± 1.6	24.5 ± 2.1	26.2 ± 1.5	18.0 ± 1.7
Liver				
Weight (g/100g body weight)	5.51 ± 0.11	5.54 ± 0.08	5.37 ± 0.15	5.21 ± 0.13
Cholesterol (mg/g)	23.0 ± 1.9	19.3 ± 1.7	28.9 ± 2.3[e]	17.8 ± 2.8
Triglyceride (mg/g)	42.0 ± 6.5	30.8 ± 3.5	59.9 ± 8.0[e]	32.6 ± 4.0

[a]Weight gain for 29 and 32 days for cholesterol-free and cholesterol-enriched diets, respectively.
[b]Mean ± SE of 8-9 rats per group.
[c]Mg per wet weight.
[d,e]Analysis of significance was made between the same fat level and between different levels of the same fat. The superscript letters d and e indicate significant difference ($P<0.05$) to 5% OL group and to 20% PHC group, respectively.

TABLE 3

Effects of Dietary Fats on Biliary Cholesterol and Bile Acids

Biliary steroids	5% Fat		20% Fat	
	OL	PHC	OL	PHC
Cholesterol-free diets				
Bile flow (ml/hr)	0.61 ± 0.04[a]	0.54 ± 0.04	0.59 ± 0.07	0.47 ± 0.06
Cholesterol (mg/ml)	0.14 ± 0.01	0.13 ± 0.01	0.18 ± 0.01[d]	0.15 ± 0.01
Bile acids				
Concentration (mg/ml)[b]	12.0 ± 1.2	12.3 ± 1.6	12.8 ± 2.2	11.7 ± 2.2
Composition (%)[c]				
Lithocholic	14.7 ± 4.8	9.5 ± 1.0	6.9 ± 1.6	14.4 ± 1.9
Deoxycholic	4.0 ± 0.6	3.3 ± 0.4	3.0 ± 0.9	3.7 ± 0.4
Chenodeoxycholic	2.3 ± 0.7	3.8 ± 0.7	4.5 ± 0.6	2.4 ± 0.6
Cholic + α-muricholic	34.5 ± 5.8	45.5 ± 4.3	51.5 ± 2.9	44.1 ± 4.1
12-Ketolithocholic	8.7 ± 3.0	7.8 ± 1.1	13.7 ± 2.5	10.4 ± 1.9
β-Muricholic	12.5 ± 5.4	13.1 ± 3.4	8.4 ± 3.1	8.0 ± 2.5
ω-Muricholic	3.0 ± 1.8	3.2 ± 1.5	1.1 ± 0.7	4.2 ± 2.0
Cholesterol-enriched diets				
Bile flow (ml/hr)	0.76 ± 0.06	0.75 ± 0.11	0.74 ± 0.13	0.83 ± 0.13
Cholesterol (mg/ml)	0.15 ± 0.01	0.14 ± 0.01	0.22 ± 0.04	0.16 ± 0.02
Bile acids				
Concentration (mg/ml)[b]	14.7 ± 1.7	17.2 ± 2.6	18.4 ± 3.1	18.0 ± 3.5
Composition (%)[c]				
Lithocholic	9.6 ± 2.8	9.8 ± 1.7	14.7 ± 2.3	10.4 ± 2.2
Deoxycholic	1.3 ± 0.2	2.1 ± 0.5	3.1 ± 0.7	0.8 ± 0.2
Chenodeoxycholic	3.9 ± 0.5	5.3 ± 1.0[e]	3.5 ± 0.5	2.7 ± 0.5
Cholic + α-muricholic	30.2 ± 1.7	27.7 ± 2.5	36.7 ± 4.3	27.7 ± 2.0
12-Ketolithocholic	11.7 ± 1.5	15.3 ± 1.6	15.2 ± 1.0	13.5 ± 1.2
β-Muricholic	25.4 ± 2.5	18.2 ± 2.2	7.9 ± 1.3[d]	15.1 ± 3.8
ω-Muricholic	1.3 ± 0.7	2.7 ± 0.7	1.1 ± 0.5	2.3 ± 0.5

[a]Mean ± SE of 5-8 rats per group.
[b]As taurocholate.
[c]Excludes 2-3 unidentified steroids.
[d,e]Significant difference ($P<0.05$) to 5% OL group and to 20% PHC group, respectively. See footnote of Table 2.

TABLE 4

Effect of Dietary Fats on Fecal Excretion of Neutral and Acidic Steroids of Rats Fed Cholesterol-Free Diets

Fecal steroids	5% Fat		20% Fat	
	OL	PHC	OL	PHC
Stool weight (g/day)[a]	0.74 ± 0.04[b]	0.71 ± 0.06	0.68 ± 0.06	0.84 ± 0.07
Neutral steroids				
Daily excretion (mg/day)	6.06 ± 0.29	7.43 ± 0.53[e]	7.38 ± 1.04[e]	10.8 ± 1.1
Concentration (mg/g)	8.30 ± 0.24	10.7 ± 0.6[de]	10.5 ± 0.7[de]	14.1 ± 1.4
Composition (%)				
Coprostanol	71.8 ± 6.6	64.0 ± 6.3[e]	48.5 ± 9.5[d]	28.6 ± 5.2
Cholesterol	28.2 ± 6.6	36.0 ± 6.3[e]	51.5 ± 9.5[d]	71.4 ± 5.2
Acidic steroids				
Daily excretion (mg/day)	2.64 ± 0.50	4.30 ± 0.50	2.37 ± 0.46[e]	6.58 ± 1.30
Concentration (mg/g)	3.70 ± 0.83	6.16 ± 0.56[d]	3.25 ± 0.35[e]	7.74 ± 1.67
Composition (%)[c]				
Lithocholic	29.4 ± 3.2	25.9 ± 3.3[e]	37.9 ± 4.3[d]	35.1 ± 2.5
Deoxycholic	7.6 ± 2.0	8.3 ± 1.5	5.5 ± 2.0	6.4 ± 1.1
Cholic + α-muricholic	21.1 ± 3.8	19.6 ± 4.9	9.6 ± 1.1[d]	13.5 ± 1.8
12-Ketolithocholic	33.2 ± 3.4	33.1 ± 2.5	38.3 ± 3.1	37.2 ± 2.2
β-Muricholic	-	-	-	-
ω-Muricholic	8.2 ± 2.9	13.6 ± 4.5	7.8 ± 2.5	6.1 ± 1.8

[a]Lyophilized feces.
[b]Mean ± SE of 8-9 rats per group.
[c]Excludes 2-3 unidentified steroids.
[d,e]Significant difference ($P<0.05$) to 5% OL group and to 20% PHC group, respectively. See footnote of Table 2.

terol to coprostanol was reduced significantly when the dietary fat level was increased (in particular with *trans* fat). Bile acid excretion was also greater in rats on *trans* fat than in those on the corresponding *cis* fat. Although bile acid excretion was enhanced by *trans* fat, it was not affected significantly by the amount of fat. *Cis* fat did not influence the fecal output of acidic steroids even when the dietary level was raised 4-fold. The amounts, rather than the types, of dietary fat appeared to influence fecal bile acid composition.

On feeding cholesterol-enriched diets (Table 5), the *trans* fat-induced increase in fecal neutral steroid excretion was apparent only when rats were fed 20% fat. Excretion of neutral steroids decreased on high fat diets (in particular the *cis* fat diet), compared to low fat diets. The ratio of coprostanol to cholesterol was comparable in 2 types of low fat diets, whereas it was reduced on the high *trans* fat diet from that on the corresponding *cis* fat diet, due to an increased transformation with the latter diet. The effects of dietary fat types on fecal excretion of bile acids were not clear, but excretion tended to be lower on high fat diets. The compositions of fecal bile acids were too varied to draw a conclusion.

Fatty Acid Compositions of Tissue Lipids

In both trials, percentages of linoleic and arachidonic acids in post-drainage serum, liver and adipose tissue lipids were similar at either level of *cis* and *trans* fat. Virtually no eicosatrienoic acid (20:3n9) was detected in any specimen. The contents of *trans*-octadecenoate in these lipids reflected the dietary levels; in rats fed cholesterol-free diets, *trans*-octadecenoate corresponded, in percentage of total fatty acids, to 12-13% in serum and liver and 25% in the adipose tissue for a 20% fat diet, and 3-4% in serum and liver and 7.5% in the adipose tissue for a 5% fat diet. Other types of *trans* fatty acids such as *t*-16:1, *ct*-18:2 or *tc*-18:2 were present only in trace amounts. These results were also reproducible in rats fed cholesterol-enriched diets.

DISCUSSION

There is a considerable divergence of opinion regarding the effect of dietary *trans* fats on plasma cholesterol levels and hence atherogenesis in humans and experimental animals (1,3). The effect appears to depend on the level of linoleic acid simultaneously supplied, as well as on the type and level of *trans* fat. In Wistar rats fed 10% fat diets containing equivalent linoleic acid (1.7 energy %), *trans*-octadecenoate, the prototype of *trans* fatty acids in the commercial hydrogenated products, was in no way hypercholesterolemic compared to the *cis* counterpart; rather it gave somewhat lower serum cholesterol levels than did the *cis*-monoenoate (18). When precautions against the linoleic acid content were not taken, the partially hydrogenated soybean oil was more hypercholesterolemic than native soybean oil (19). In the present study with Sprague-Dawley rats, *trans* fat was at least not hypercholesterolemic at both high and low dietary levels. The somewhat less clear response of the serum cholesterol level in the present study may be ascribed to the difference in the strain of rats and in the supply of linoleic acid.

The effects of *trans* fat on serum levels of apolipoproteins were variable, but the reduction of the apo B level, due to feeding a 20% *trans* fat diet high in cholesterol, compared to the corresponding *cis* fat diet, was a phenomenon previously observed in rats fed 10% fat diets containing cholesterol (18). The triglyceride level of post-drainage serum also tended to be reduced on a 20% *trans* fat diet, compared to a 20% *cis* fat diet (89.8 ± 6.3 vs 148 ± 34 mg/dl). It seems that *trans*-octadecenoate, compared to the *cis* counterpart, specifically modifies the metabolism of triglyceride-rich lipoproteins.

Irrespective of the presence or absence of cholesterol in the diets, there were no consistent differences in concentrations of biliary cholesterol and bile acids when rats were maintained on the diet containing the same level of dietary fat. The compositions of biliary bile acids changed to a considerable extent depending on the type of dietary fat, but the variability of the results rendered the evaluation of the difference difficult.

In rats fed diets either free of, or containing, cholesterol, fecal excretion of neutral steroids increased on feeding the high *trans* fat diets, compared to the high *cis* fat diets, whereas it was comparable on low-fat diets. We have previously observed an increase in the fecal output of neutral steroids in rats fed diets containing 10% partially hydrogenated corn oil (18). It is thus likely that *trans*-octadecenoate, at concentrations above a certain dietary level, stimulates the fecal elimination of neutral steroids. Along with this increase was a lesser magnitude of transformation of cholesterol to coprostanol, suggesting a specific alteration of the flora of the intestinal bacteria. Dietary factors, however, appear to have a limited effect on the bacterial flora in the colon (21). In this connection, the apparent absorption rate (22) of dietary fat was slightly but clearly lower with *trans* fat than with *cis* fat; with cholesterol-free diets, the apparent absorption rates were 91.6 and 92.8% for 5 and 20% *trans* fat diets and 95.0% and 97.2% for the corresponding *cis* fat diets. Almost the same results were obtained with cholesterol-enriched diets.

It is interesting that, in rats fed cholesterol-enriched diets, high dietary *cis* fat compared to low *cis* fat reduced fecal neutral steroid excretion, suggesting increased absorption of cholesterol. In

TABLE 5

Effect of Dietary Fats on Fecal Excretion of Neutral and Acidic Steroids of Rats Fed Cholesterol-Enriched Diets

Fecal steroids	5% Fat		20% Fat	
	OL	PHC	OL	PHC
Stool weight (g/day)[a]	1.03 ± 0.05[b]	1.00 ± 0.05	0.82 ± 0.04	0.95 ± 0.08
Neutral steroids				
Daily excretion (mg/day)	74.0 ± 4.5	69.2 ± 4.0[e]	40.8 ± 2.4[d]	59.5 ± 3.5
Concentration (mg/g)	71.7 ± 1.7	69.4 ± 2.1[e]	49.8 ± 0.9[de]	63.6 ± 2.3
Composition (%)				
Coprostanol	22.1 ± 3.5	21.0 ± 3.9	46.5 ± 7.0[e]	16.1 ± 3.2
Cholesterol	77.9 ± 3.5	79.0 ± 3.9	53.5 ± 7.0[e]	83.9 ± 3.2
Acidic steroids				
Daily excretion (mg/day)	8.11 ± 1.07	9.23 ± 1.11[c]	6.03 ± 0.83	6.59 ± 1.18
Concentration (mg/g)	7.88 ± 1.06	9.29 ± 1.09	7.59 ± 1.11	7.11 ± 1.11
Composition (%)				
Lithocholic	22.3 ± 4.7	27.2 ± 4.6	20.2 ± 2.8	23.0 ± 3.3
Deoxycholic	-	2.1 ± 0.7	9.2 ± 2.5	7.1 ± 4.4
Cholic + α-muricholic	36.5 ± 5.4	13.8 ± 4.4	6.7 ± 1.3[d]	16.6 ± 4.6
12-Ketolithocholic	7.2 ± 3.2	15.9 ± 4.7	13.1 ± 3.5	10.1 ± 6.7
β-Muricholic	21.6 ± 4.2	13.3 ± 4.0	13.3 ± 2.5	32.5 ± 6.6
ω-Muricholic	12.0 ± 3.3	25.3 ± 7.3	35.3 ± 4.5	9.7 ± 1.8

[a]Lyophilized feces.
[b]Mean ± SE of 8-9 rats per group.
[c]Excludes 2-3 unidentified steroids.
[d,e]Significant difference ($P<0.05$) to 5% OL group and to 20% PHC group, respectively. See footnote of Table 2.

fact, the livers from rats fed a 20% *cis* fat diet, compared to a 5% *cis* fat diet, contained a high level of cholesterol.

Since cholesterol lost to the gut, either dietary or endogenously synthesized, appears to have a role in large bowel cancer (14,23), the observed effects of *trans* fat are of considerable importance. However, *trans* fat at the lower level showed no clear effect on cholesterol excretion. This group of rats was fed ca. 25% of dietary fatty acids as *trans*-octadecenoate (compared to 40% in the case of a 20% fat diet). This level is markedly higher than the corresponding value in diets ingested by humans in North America (4,24). In addition, bile acids (25,26), in particular secondary bile acids (27,28), have also been considered to be related to the development of colon tumor. *Trans* fat at either level enchanced fecal bile acid output when cholesterol-free diets were fed to rats and the dose-dependent increase in fecal bile acid was observed only with this isomer. In both trials, however, the proportion of secondary bile acids remained apparently unchanged. At present, therefore, no evidence is available to correlate *trans*-fat intake and colon carcinogenesis (4,5).

In conclusion, the present results, together with those obtained in the preceding study (18), strongly suggest that *trans*-octadecenoate, compared to *cis*-octadecenoate, is not hypercholesterolemic. Although the *trans*-monoene in amounts above a certain dietary level leads to an increased loss of fecal steroids, this phenomenon may be connected with a lower absorption rate of dietary *trans* fat itself.

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REFERENCES

1. Applewhite, T.H. (1981) J. Am. Oil Chem. Soc. 58, 260-269.
2. Kinsella, J.E., Bruckner, G., Mai, J., and Shimp, J. (1981) Am. J. Clin. Nutr. 34, 2307-2318.
3. Kritchevsky, D. (1982) Fed. Proc. 41, 2813-2817.
4. Enig, M.G., Munn, R.J., and Keeney, M. (1978) Fed. Proc. 37, 2215-2220.
5. Enig, M.G., Munn, R.J., and Keeney, M. (1979) Fed. Proc. 38, 2437-2439.
6. Applewhite, T.H. (1979) Fed. Proc. 38, 2435.
7. Bailar, J.C. (1979) Fed. Proc. 38, 2435-2436.
8. Meyer, W.H. (1979) Fed. Proc. 38, 2436-2437.
9. Reddy, B.S., Mangat, S., Sheinfil, A., Weisburger, J.H., and Wyner, E.L. (1977) Cancer Res. 37, 2132-2137.
10. Carroll, K.K. (1980) J. Env. Pathol. Toxicol. 3, 253-271.
11. Awad, A.B. (1981) J. Natl. Cancer Inst. 67, 189-192.
12. Awad, A.B. (1982) J. Natl. Cancer Inst. 69, 320-321.
13. Hunter, J.E. (1982) J. Natl. Cancer Inst. 69, 319-320.
14. Broitman, S.A. (1981) Cancer Res. 41, 3738-3740.
15. Cohen, B.I., and Raicht, R.F. (1981) Cancer Res. 41, 3759-3760.
16. Reddy, B.S. (1981) Cancer Res. 41, 3700-3705.
17. Reddy, B.S. (1981) Cancer Res. 41, 3766-3768.
18. Sugano, M., Watanabe, M., Kohno, M., Cho, Y.-J., and Ide, T. (1983) Lipids 18, 375-381.
19. Sugano, M., Ide, T., Kohno, M., Watanabe, M., Cho, Y.-J., and Nagata, Y. (1983) Lipids, 18, 186-192.
20. Snedecor, G.W., and Cochran, W.G. (1967) in Statistical Methods, 6th edn., pp. 472-503, The Iowa State University Press, Ames, IA.
21. Kay, R.M. (1981) Cancer Res. 41, 3774-3777.
22. Kamer, J.H., Bokkel Huinink, H., and Weyers, H.A. (1949) J. Biol. Chem. 177, 347-355.
23. Cruse, J.P., Lewin, M.R., and Clark, C.G. (1979) Lancet i, 152-155.
24. Kummerow, F.A. (1975) J. Food Sci. 40, 12-17.
25. Narisawa, T., Magadia, N.E., Weisburger, J.H., and Wynder, E.L. (1974) J. Natl. Cancer Inst. 55, 1093-1097.
26. Koga, S., Kaibara, N., and Takeda, R. (1982) Cancer 50, 543-547.
27. Kawalek, J.C., and Andrews, A.W. (1977) Fed. Proc. 36, 844.
28. Cohen, B.I., Raicht, R.F., Deschner, E.E., Takahashi, M., Sarwal, A.N., and Fazzini, E.J. (1979) J. Natl. Cancer Inst. 64, 573-578.

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Subcellular Localization of Triacylglycerol Synthesis in Spinach Leaves

B.A. MARTIN and **R.F. WILSON***, *USDA-ARS, Crop Science Department, North Carolina State University, Raleigh, NC 27650*

ABSTRACT

The subcellular location of diacylglycerol acyltransferase (EC 2.3.1.20) in spinach leaves (*Spinacia oleracea* L. cv. Longstanding Bloomsdale) was determined after sucrose-gradient centrifugation of tissue homogenates. Enzyme activity was associated primarily with gradient fractions containing oil bodies and intact chloroplasts. Gradient fractions enriched with the endoplasmic reticulum contained insignificant levels of diacylglycerol acyltransferase activity. On the basis of chlorophyll, diacylglycerol acyltransferase activity in crude homogenates was not significantly different from that in intact chloroplasts after Percoll density-gradient centrifugation. Among membrane fractions isolated from hypotonically treated chloroplasts, envelopes contained the greatest diacylglycerol acyltransferase and galactosyltransferase activity. These data demonstrated that chloroplasts were a subcellular site for triacylglycerol biosynthesis in spinach leaves.

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INTRODUCTION

Triacylglycerol (TG) is an important form of storage lipid in many plant tissues (1). The pathway for TG biosynthesis appears to be similar in plant species; however, the subcellular location of diacylglycerol acyltransferase (DGAT), the enzyme that catalyses TG biosynthesis, is not well defined (2). Evidence from electron micrographs suggests that vesicles (sphereosomes) detached from terminal strands of the endoplasmic reticulum (ER) develop into oil bodies (3). The implication is that DGAT is associated with the ER and is transferred to oil bodies by the membrane-bounding sphereosomes. Although oil bodies isolated from developing castor beans (4) and crambe seed (5) may synthesize TG from [^{14}C]acyl-CoA or [^{14}C]glycerol-3-phosphate, no direct biochemical proof was found that oil bodies are formed from sphereosomes. In fact, Gurr concludes that sphereosomes do not develop into lipid bodies and that each entity has separate but distinct function (2). Ichihara (6) also contends that the origin of lipid bodies is independent of the ER in maturing safflower seed.

Given that sphereosomes are formed from the ER, the controversy over oleosome formation may abate with information on the subcellular location of DGAT activity. From previous reports, significant levels of TG synthetic activity are sedimented at 3,000 *g* from maturing safflower-seed homogenates (7) and at 3,000 *g* and 20,000 *g* from homogenates of spinach leaves (8). Although the differential centrifugation fractions could have been contaminated with ER, TG synthesis from [^{14}C]acetate is reported in pea plastids (9) and in purified spinach chloroplasts (10). Hence, in certain plant tissues, DGAT may be found in organelles other than the ER.

The purpose of this study was to find direct evidence for associating DGAT activity with the ER and/or chloroplasts from spinach leaves. Spinach leaves contain relatively low levels of TG; however, that tissue source was used in the first report from our laboratory on isolating and characterizing DGAT (8). The DGAT assay developed in that work was used with sucrose and Percoll gradient centrifugation methods to fractionate cytoplasmic organelles. The findings of this investigation have revealed that DGAT activity is associated with chloroplast envelopes from spinach leaves.

*To whom correspondence should be addressed.

MATERIALS AND METHODS

Preparing Tissue

Spinach (*Spinacia oleracea* L. cv. Longstanding Bloomsdale) plants were grown in a greenhouse. Fully expanded leaves were harvested and chilled to 4 C. Leaf tissue (10 g fresh weight) was chopped with an electric razor knife in 20 ml of buffered grinding medium. The grinding medium contained: 0.4 M sucrose; 50 mM Bicine [N,N-*bis*(2-hydroxy-ethyl glycine)], pH 7.6; 1 mM KCl; 0.1 mM $MgCl_2$; 1 mM NA-EDTA. The homogenate was squeezed through 6 layers of cheesecloth and one layer of Miracloth. A 7 ml aliquot of the filtrate was applied to a step gradient of 3 ml 2.3 M sucrose, 3 ml 1.9 M sucrose, 4 ml 1.8 M sucrose, 4 ml 1.75 M sucrose, 4 ml 1.7 M sucrose, 4 ml 1.5 M sucrose, 3 ml 1.3 M sucrose, 3 ml 1 M

sucrose, and 3 ml 0.83 M sucrose. Each gradient was replicated 8 times. All sucrose solutions were prepared in a medium containing 10 mM Bicine, pH 7.6; 1 mM NA-EDTA; 0.1 mM $MgCl_2$; 1 mM KCl.

The gradients were centrifuged at 4 C in a Beckman L80 centrifuge equipped with a Beckman SW28 rotor. The centrifugation was conducted at 141,000 *g* for 4 hr. The w^2t value applied to all gradients was 1.22×10^{11}. An ISCO 185 density gradient fractionator was used to collect 2 ml fractions from the top of the gradients. All fractions were stored at 4 C before the enzyme assay.

Localizing Organelles

All gradient fractions were assayed for enzymes characteristic of specific organelles. Catalase activity, determined by the decrease in A at 240 nm (11), indicated the location of peroxisomes. Regions of the gradient containing ER were determined by measuring the NADH-dependent increase in A at 550 nm for antimycin A insensitive NADH-Cyt C reductase activity (12). Cyt C oxidase activity associated with mitochondrial inner membranes was determined by a decrease in A of reduced Cyt C at 550 nm (13). In the latter case, the fractions were incubated with 10 μl digitonin for 1 min before the addition of the buffer and substrate. RuBP carboxylase activity was determined by the incorporation of $^{14}CO_2$ into 3-phosphoglyceric acid (14). DGAT activity was determined by the incorporation of [^{14}C] 18:1-CoA into TG (8). Galactosyltransferase (GALT) activity associated with chloroplast envelopes was determined by incorporation of UDP-[^{14}C]galactose into glycolipids (15). Chlorophyll (chl) was determined by A at 663 and 645 nm (16); protein was determined by a modified Lowry procedure (17). All data represent mean values from all gradients performed.

Preparing Intact Chloroplasts

Spinach leaves (200 g fresh weight) were homogenized in 1 l grinding medium, as described earlier. Intact chloroplasts were isolated on a 10-80% (w/v) Percoll gradient (18). The chloroplasts were washed twice with a grinding medium, centrifuged at 2,000 *g* for 2 min and resuspended in the grinding medium. A fraction of those preparations was osmotically shocked in 50 mM Bicine, pH 7.6. Intact and osmotically shocked chloroplasts were adjusted to an equal concentration, 0.5 mg chl$(ml)^{-1}$, before incubation at 25 C for 30 min with 675 BAEE units of DPCC-treated trypsin (Sigma Chemical Co., St. Louis, MO) in a total volume of 0.25 ml. At the end of treatment, a 10-fold excess of trypsin inhibitor (soybean) was added to all treatments and controls.

Preparing Chloroplast Envelopes

Purified chloroplasts, prepared as described above, were resuspended in 28 ml swelling medium containing 10 mM Bicine, pH 7.6, and 4 mM $MgCl_2$. Equal portions of the preparation (14 ml) were loaded onto step gradients (19) containing: 6 ml 1.5 M sucrose, 6 ml 1.2 M sucrose, 6 ml 0.93 M sucrose and 6 ml 0.6 M sucrose. The gradients were centrifuged at 4 C in a Beckman L80 centrifuge equipped with a Beckman SW28 rotor for 1 hr at 141,000 *g*. Each set of gradients was replicated 4 times. four fractions were collected from the top of a gradient as before; the volume of fraction 1 was 14 ml and the volumes for fractions 2 to 4 were 6 ml each. Each fraction volume was increased to 38 ml with the swelling medium and centrifuged at 4 C for 1 hr at 141,000 *g*. The pellets were resuspended in 1 ml swelling buffer. Total chl, protein, and GALT, DGAT, Cyt C reductase, and Cyt C oxidase activities were determined as stated above.

Lipid Analysis

Whole spinach leaves (10 g fresh weight) were homogenized consecutively with a Brinkman Polytron and a Ten-Broeck tissue grinder in 40 ml chloroform/methanol (2:1 v/v). The homogenate was filtered with an additional 20 ml chloroform/methanol (2:1 v/v) and 30 ml methanol. After filtration, 50 ml of deionized water was added to the filtrate. The mixture was shaken and centrifuged at 1,000 *g* for 20 min to form a biphasic solution. The phase containing lipids was dried in vacuo. Lipids were extracted from a 1 ml portion of 4 separate, intact chloroplast preparations by adding 4 ml chloroform/methanol (1:1, v/v). The mixture was vortexed and centrifuged at 1,000 *g* for 10 min. The phase containing lipids was dried under N_2. Fat layers (oil bodies) obtained from the 4 sucrose gradients were extracted by the method described for intact chloroplasts. All lipid extracts were redissolved in chloroform/methanol (2:1, v/v). Total polar lipid (TPL), diacylglycerol (DG), and TG were separated from the total lipid extract by TLC on precoated Absorbosil-Plus 5 plates (Applied Science Lab., State College, PA) with petroleum ether/diethyl ether/glacial acetic acid (80:20:0.8, v/v/v) as the developing solvent. Lipid classes were identified by parallel chromatography with authentic phosphatidylcholine, DG, and TG standards (Sigma Chemical Co.,

St. Louis, MO). Lipid classes were quantitatively analyzed by GC as described previously (20). Radioactive TG from DGAT reactions was isolated by cochromatography with a mixture of TG, sterylester, and free fatty acid standards (8).

RESULTS AND DISCUSSION

The activities of marker enzymes for subcellular organelles from spinach leaves, chl, protein, and DGAT activity within sucrose-gradient fractions were calculated on a volume basis (Figs. 1-2). The ER, identified by antimycin A insensitive NADH-Cyt C reductase activity, banded at a density of ca. 1.13 $g(cc)^{-1}$, fractions 4-6; mitochondria, located by Cyt C oxidase activity, had a density of ca. 1.18 $g(cc)^{-1}$, fractions 7-9; intact chloroplasts, indicated by RuBP-carboxylase activity, banded at 1.22 $g(cc)^{-1}$, fractions 10 to 11; and peroxisomes, distinguished by catalase activity, had density of ca. 1.24 $g(cc)^{-1}$, fractions 15 to 16. The supernatant of the gradient (fractions 1 to 3) contained significant levels of RuBP-carboxylase and catalase activity because the disruption of intact organelles could not be prevented. The major chlorophyll bands also indicated the location of broken chloroplasts (fraction 7) and intact chloroplasts (fractions 10 and 11).

The major peaks for DGAT activity were found at sucrose-gradient densities of ca. 1.08 $g(cc)^{-1}$ and 1.22 $g(cc)^{-1}$. These activities did not coincide with regions of the gradient enriched with ER. Hence, these data cast further doubt on the speculation that oil bodies were formed from the ER. DGAT activity was coincident with gradient fractions containing intact chloroplasts, yet insignificant levels of DGAT activity were found in fractions containing broken chloroplasts (presumably thylakoids or grana). The work of Shine et al. (21) clearly demonstrated that spinach grana did not contain DGAT activity. In addition, low levels of DGAT activity in the supernatant fraction containing RuBP-carboxylase activity suggested that chloroplastic DGAT was not a stromal enzyme. By deduction, one might then presume that DGAT in intact chloroplasts was associated with the chloroplast envelopes. The density of chloroplast envelopes (22) was reported to be about 1.08 $g(cc)^{-1}$, the density at which oil bodies were found in our gradients. We have not been able to devise a definitive means for distinguishing whether the DGAT activity found in the fat layer was attributable to the oil bodies per se or to the presence of chloroplast envelopes. We could, however, attempt to prove that the proposed association between DGAT and chloroplast envelopes was valid.

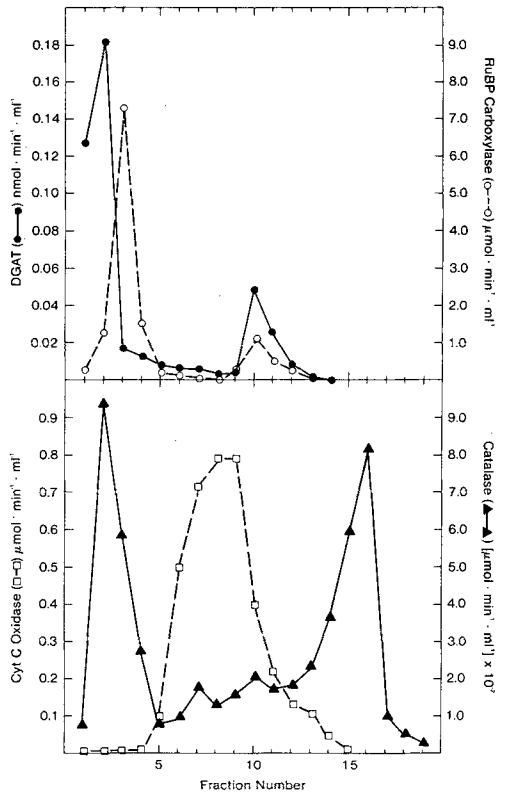

FIG. 1. Distribution of organelle marker enzymes in sucrose density gradients. RuBP carboxylase (○—○), Cyt C oxidase (□—□), catalase (▲—▲), and DGAT (●—●) from spinach leaf homogenates applied directly to a sucrose step gradient and centrifuged as described in *Methods*. Enzyme activities were expressed on a volume basis. All fractions were 2 ml.

Intact chloroplasts, isolated on Percoll density gradients and washed twice with a grinding medium, contained similar levels of DGAT or GALT activity on a chl basis, as did crude extracts (Table 1). These chloroplast preparations contained insignificant levels of Cyt C oxidase or Cyt C reductase activity. Although DGAT or GALT activity was lower when intact chloroplasts were lysed, tryptic digestion did not significantly affect the activity of either enzyme. These data suggested that both enzymes, or at least their active sites, were embedded within the chloroplast membranes. To show the proposed association of DGAT with chloroplast envelopes, purified intact chloroplast preparations were hypotonically disrupted (19) and fractionated by discontinuous sucrose-gradient centrifugation (23). Fractions enriched with stromal enzymes, envelopes, plastoglobuli, and thylakoids were characterized as described (23). The activity

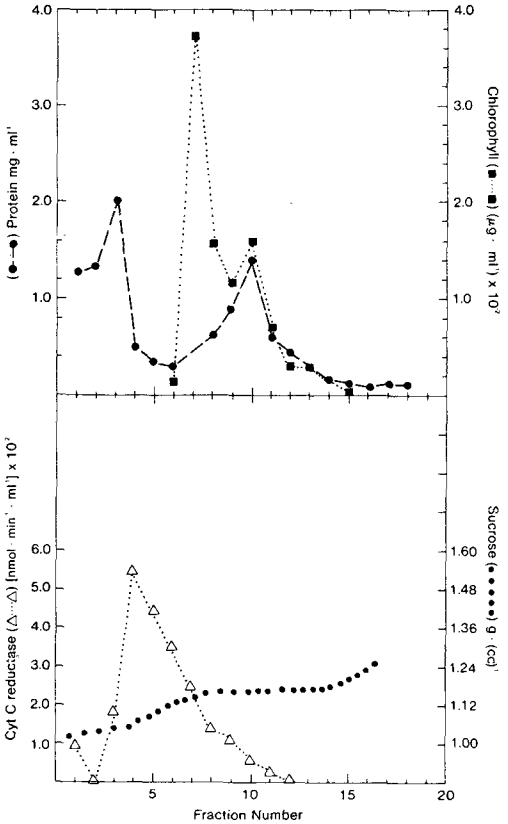

FIG. 2. Distribution of organelle marker enzymes in sucrose density gradients. Sucrose density (.....), protein (●—●), Cyt C reductase (△—△), and chlorophyll (■—■). Enzyme activity was expressed on a volume basis. All fractions were 2 ml.

of marker enzymes for the ER and mitochondria were negligible among these chloroplast membrane fractions. The distribution of DGAT activity among the fractions verified that DGAT was not a stromal enzyme and revealed that the greatest activity was associated with chloroplast envelopes (Table 2). Because the distribution of GALT activity paralleled DGAT activity among the fractions, the DGAT activity found with plastoglobuli and thylakoids was attributed to contamination by chloroplast envelopes. Hence, these data suggest that chloroplastic DGAT could be exclusively associated with chloroplast envelopes.

TABLE 1

Effect of Trypsin upon DGAT and GALT Activity

Preparation[a]	Trypsin[b]	DGAT	GALT
		-nmol $(min)^{-1}$ $(mg\ ch)^{-1}$ -	
Intact chloroplasts	+	0.336	0.525
	−	0.386	0.615
Lysed chloroplasts	+	0.257	0.265
	−	0.290	0.355
Crude extract	−	0.039	0.640
LSD 0.05		0.079	0.120

[a]Chloroplasts were isolated on Percoll density gradients; a portion of the preparation was lysed by osmotic shock. All preparations were adjusted to a final concentration of 0.5 mg chl$(ml)^{-1}$ before trypsin treatment.

[b]675 BAEE units of DPCC-treated trypsin was added to each preparation as indicated. After 30 min at 25 C, a 10-fold excess of trypsin inhibitor was added to arrest trypsin activity. Trypsin inhibitor per se had no effect upon DGAT or GALT activity.

Intact chloroplasts, after Percoll gradient centrifugation, contained 67.8 ± 1.2 μmol TG (mg chl)$^{-1}$; in addition, the fatty acid composition of chloroplastic TG was not statistically different from cytoplasmic TG (Table 3). The association of DGAT activity with chloroplasts, plus the evidence that chloroplasts also have enzymes necessary for acyl-CoA and DG formation (24-27), have provided both a means and a method for TG biosynthesis in chloroplasts. Chloroplastic TG could then be a constituent of the osmiophilic bodies often observed within chloroplasts from higher plants (28). The biological relevence of these findings will be tested in studies with plastids from developing oilseeds.

TABLE 2

Distribution of DGAT and GALT Activity Among Membrane Fractions from Hypotonically Disrupted Chloroplasts

Fraction	Protein	Chlorophyll	GALT	DGAT
	----------mg$(ml)^{-1}$----------		----nmol $(min)^{-1}$ $(ml)^{-1}$----	
Stroma	1.4	ND[a]	0.05	0.09
Envelopes	1.6	ND[a]	2.29	1.14
Plastoglobuli	1.8	0.028	1.04	0.83
Thylakoids	40.0	6.693	0.95	0.37
LSD 0.05			0.39	0.20

[a]Not detectable.

TABLE 3

Triacylglycerol Composition of Preparations from Spinach Leaves

Sample	Fatty acid						Total
	16:0	16:1	18:0	18:1	18:2	18:3	
	mol %						nmol TG (mg chl)$^{-1}$
Whole leaf[a]	21.5	2.8	6.0	16.5	22.5	30.7	246.3 ± 4.1
Intact chloroplast[b]	23.1	2.2	7.1	17.1	20.8	29.7	67.8 ± 1.2
Fat layer[c]	20.6	2.9	5.8	16.2	23.5	31.0	138.6 ± 3.0
LSD 0.05	3.0	0.9	1.7	1.1	3.2	1.6	

[a]Spinach leaves contained 1.4 mg chl(g fresh wt)$^{-1}$.
[b]From Percoll density gradient centrifugation.
[c]TG content of the fat layer was calculated on a fresh weight basis; nmol TG (mg chl)$^{-1}$ was extrapolated from the mg chl(g fresh weight)$^{-1}$ (leaf)$^{-1}$.

ACKNOWLEDGMENTS

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REFERENCES

1. Appelquist, L.A. (1975) in Recent Advances in the Chemistry and Biochemistry of Plant Lipids (Galliard, T., and Mercer, E.I., eds.) pp. 247-286, Academic Press, New York.
2. Gurr, M.I. (1980) in The Biochemistry of Plants (Stumpf, P.K., and Conn, E.E., eds.) Vol. 4 pp. 205-248, Academic Press, New York.
3. Frey-Wyssling, A., Grieshaber, E., and Muhlethaler, K. (1963). J. Ultrastruct. Res. 8, 506-516.
4. Harwood, J.L., Sodja, A., Stumpf, P.K., and Stumpf, A.R. (1971) Lipids 6, 851-854.
5. Gurr, M.I., Blades, J., Appleby, R.S., Smith, C.G., Robinson, M.P., and Nichols, B.W. (1974) Eur. J. Biochem. 43, 281-290.
6. Ichihara, K. (1982) Agric. Biol. Chem. 46, 1767-1773.
7. Ichihara, K., and Noda, M. (1981) Phytochemistry 20, 1245-1249.
8. Martin, B.A., and Wilson, R.F. (1983) Lipids 18, 1-6.
9. Panter, R.A., and Boardman, N.K. (1973) J. Lipid Res. 14, 667-671.
10. Dubacq, J-P., Drapier, D., and Tremolieres, A. (1983) Plant Cell Physiol. 24, 1-9.
11. Luck, H. (1965) in Methods of Enzymatic Analysis (Bergmeyer, H.U., ed.), pp. 885-894, Academic Press, New York.
12. Bowles, D.S., and Kauss, H. (1976) Biochim. Biophys. Acta 443, 360-374.
13. Smith, L. (1958) in Methods of Biochemical Analysis (Glick, D. ed.) 2d ed., Vol. 2, pp. 427-435, Interscience Publishers, Inc., New York.
14. McCurry, S.D., Hall, N.P., Pierce, J., Paech, C., and Tolbert, N.E. (1978) Biochem. Biophys. Res. Commun. 84, 895-900.
15. Douce, R., and Joyard, J. (1980) Methods Enzymol. 69, 290-301.
16. Arnon, D.I. (1949) Plant Physiol. 24, 1-15.
17. Bensadoun, A., and Weinstein, D. (1976) Anal. Biochem. 70, 241-250.
18. Morganthaler, J.J., Price, C.A., Robinson, J.M., and Gibbs, M. (1974) Plant Physiol. 54, 532-534.
19. Douce, R., Holtz, B. and Benson A. (1973) J. Biol. Chem. 248, 7215-7222.
20. Wilson, R.F., Rinne, R.W., and Brim, C.A. (1976) J. Am. Oil Chem. Soc. 53, 595-597.
21. Shine, W.E., Mancha, M., and Stumpf, P.K. (1976) Arch. Biochem. Biophys. 173, 472-479.
22. Cline, K., Andrews, J., Mersey, B., Newcomb, E.H., and Keegstra, K. (1981) Proc. Natl. Acad. Sci. USA 78, 3595-3599.
23. Joyard, J., and Douce, R. (1976) Physiol. Veg. 14, 31-48.
24. Harwood, J.L., and Stumpf, P.K. (1972) Lipids 7, 8-19.
25. Shimakata, T., and Stumpf, P.K. (1982) Plant Physiol. 69, 1257-1262.
26. Sanchez, J., and Mancha, M. (1981) Planta 153, 519-523.
27. Bertrams, M., and Heinz, E. (1976) Planta 132, 161-168.
28. Palmer, R.G., Sheridan, M.A., and Tabatabai, M.A. (1979) Cytologia 44, 881-891.

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Restriction of Maternal Food Intake Inhibits Fatty Acid Activation in Developing Rat Hearts

NIRMALA K. MENON*, GOVIND A. DHOPESHWARKAR[2] and JAMES F. MEAD,
Laboratory of Biomedical and Environmental Sciences, University of California, 900 Veteran Avenue, Los Angeles, CA 90024

ABSTRACT

We studied the effect of restricting the diet of pregnant and lactating rats on the β-oxidation of fatty acids by the developing heart in suckling pups. Control pregnant rats were fed a stock diet ad libitum. For the experimental group, food was restricted to half of the control intake on the seventh day of pregnancy and continued through lactation. The pups on the restricted diet were significantly smaller than the controls. At postnatal days 5, 14 and 21, the β-oxidation of [1-^{14}C] palmitate by heart homogenates was determined in the presence of ATP, carnitine and CoA. At day 21, the production of $^{14}CO_2$ was 60% lower in the group on the restricted diet. Consequently, the possibility of inhibiting activation or intramitochondrial transport of fatty acids by heart mitochondria was studied in vitro using [1-^{14}C] palmitate, [1-^{14}C] palmitoyl CoA and [1-^{14}C] palmitoyl carnitine. With [1-^{14}C]-palmitate, the rate of $^{14}CO_2$ produced was 2464 ± 317 cpm/mg protein/min for the control and 1682 ± 91 for the restricted diet group. With [1-^{14}C] palmitoyl CoA and [1-^{14}C] palmitoyl carnitine, the oxidation rate of the experimental group was similar to control values, showing clearly that the inhibition of oxidation was from a problem with activation. A significant decrease in palmitoyl CoA synthetase activity in the heart homogenates and mitochondria of the diet-restricted pups took place. *Lipids* 19:122-126, 1984.

INTRODUCTION

That the capacity of the heart to degrade and use fatty acids is higher during the suckling period than at the fetal or post-weaning stages in the rat (1-3) is well known. The activities of the 2 key enzymes, palmitoyl CoA synthetase and carnitine palmitoyl transferase, involved in the β-oxidation system, are also reported to peak during this period in the heart (1,4). Our earlier work suggested that maternal dietary imbalances of fatty acids can produce profound alterations in the fatty acid metabolism of the myocardium of the progeny (5). In the present study, the maternal diet was restricted to half of the ad libitum control intake from the seventh day of pregnancy according to the techniques developed by Chow and Lee (6). This technique was especially preferred over that of varying the litter size (7), to produce uniform litters by avoiding excessive over- and undernourishment in pups of the same litter. The oxidation of palmitate by the myocardium was studied under these conditions.

MATERIALS AND METHODS

Animals and Dietary Protocol

The rats used in this study were proven breeders of the Wistar strain, Crl:(Wl)BR, from Charles River Breeding Laboratories, Wilmington, MA. They were fed a stock diet (Wayne Laboratories, Chicago, IL). The composition of the diet is given in Table 1. Food consumption by the control animals was monitored and on the seventh day of pregnancy a group of 20 rats was restricted to 50% of the ad libitum control intake. Another group of 3 rats was put on a similar dietary regime on the fourteenth day of pregnancy. The underfed rats consumed their food faster than the controls. To compensate for this difference in consumption, the control group and the restricted group were fed the daily supply in divided batches. This dietary regime continued throughout pregnancy and lactation. Eight to 10 pups were maintained in each litter of the control group as well as in the diet-restricted group. One litter was used only once during the study.

On postnatal days 5, 14 and 21, pups were sacrificed by decapitation and the hearts were removed and used immediately for the respective experiments. Gastric milk and blood samples were collected from the pups. Separated serum samples and milk samples were stored at −70 C until analysis.

Chemicals

The chemicals used and their suppliers are given below:

Trizma-HCL, ATP (disodium salt), Coenzyme A (lithium salt) and Protenase from Sigma Chemical Company, St. Louis, MO.

DL-carnitine HCl and dithiothreitol from

* To whom correspondence should be addressed.
[1] Presented at the 74th Annual Meeting of the American Oil Chemists' Society, Chicago, Illinois, May 1983.
[2] Deceased.

TABLE 1

Composition of the Stock Diet

	Weight (percentage)
Carbohydrate	56.3
Protein	28.9
Fat	5.0
Fiber	4.3
Salt	5.6

The stock diet used was 'LabBlox' from Wayne Laboratories, Chicago, IL. This diet contains vitamins and minerals two or more times in excess of the recommended levels for rats by the National Academy of Sciences (8) except for the following: pyridoxine hydrocholride 5.5 mg/kg and vitamin E 35 1U/kg. Vitamin K was not present. Linoleic acid made up 46.6% of the total fatty acids of the diet (9).

Calbiochem-Behring Corporation, La Jolla, CA. Sucrose from Fisher Scientific Company, Fairlawn, NJ.

J.T. Baker Chemical Co., Phillipsburg, NJ provided KH_2PO_4, KCl and $MgCl_2 6H_2O$.

ICN, Chemicals and Nuclear Division, Irvine, CA provided [1-^{14}C]palmitate (Sp. Act. 56.8 mCi/mmole). The ammonium salt was prepared by adding a few drops of 1 M ammonia, evaporating the excess ammonia and dissolving the ammonium salt in 1% Triton solution in water.

New England Nuclear, Boston, MA provided [1-^{14}C]palmitoyl CoA (Sp. Act. 54.6 mCi/mmole) and [1-^{14}C]palmitoyl carnitine (Sp. Act. 55.3 mCi/mmole). The chemical and radiochemical purity was found to be greater than 98%. They were used as aqueous solutions.

β-Oxidation Measurements

Heart homogenates were prepared by the method reported in our previous article (5). Heart mitochondria were prepared using the method of Pande and Blanchaer (10).

The incubation mixture, a total volume of 2.5 ml, consisted of 100-500 μg protein, 100 mM sucrose, 10 mM potassium phosphate (pH 7.4), 0.1 nmole of substrate (0.1 μCi of [1-^{14}C]palmitate, [1-^{14}C]palmitoyl CoA or [1-^{14}C]palmitoyl carnitine), 2 mM ATP, 0.05 mM CoA, 0.4 mM $MgCl_2$, 0.01 mM EDTA, 80 mM KCl and 0.5 mM carnitine. Additional CoA was not added to the incubation medium when [1-^{14}C]palmitoyl CoA was the substrate. Triplicate determinations were made in a Dubnoff shaker at 37 C for 30 min. $^{14}CO_2$ was collected in Kontes cups containing 0.15 ml of 8% NaOH and counted using Aquasol (New England Nuclear, Boston, MA).

Palmitoyl CoA Synthetase (EC 6.2.1.3) Assay

The radioassay developed for the liver by Bar-Tana et al. (11) was modified for heart homogenates and mitochondria. The hearts were placed in chilled 0.25 M sucrose, washed free of blood and homogenized using a Tissuemizer (Tekmar Co., Cincinnati, OH) at setting 35 for 15-20 sec. The homogenate was centrifuged at 600 × g at 4 C; the supernatant was filtered through gauze and used for the assay.

The reagents used for the assay were 70 mM Tris-HCl buffer (pH 7.4 at 25 C), 4 mM $MgCl_2$, 5% Triton X-100, 20 mM ATP, 1.7 mM CoA, 100 mM dithiothreitol and Dole's medium containing isopropanol/n-hexane/1 M H_2SO_4(40:10:1). A reagent mixture containing 0.3 ml Tris-HCl buffer, 0.04 ml Triton, 0.2 ml $MgCl_2$, 0.2 ml ATP, 0.06 ml CoA, 0.02 ml dithiothreitol and 0.12 ml H_2O was prepared.

The assay mixture, a total volume of 0.22 ml consisting of 0.1 ml of the reagent mix, 0.02 ml [1-^{14}C]palmitate (0.1 nmole, 0.1 μCi) and 100-300 μg protein, was incubated at 30 C for 5 min. The reaction was terminated by the addition of 1 ml of Dole's medium followed by 0.4 ml of H_2O and 0.6 ml hexane. In the blanks, Dole's medium was added before proteins were added. After thorough mixing and subsequent centrifugation, the upper phase, containing the unreacted palmitate, was removed by suction. The lower phase was washed 6 times using 0.6 ml hexane each time while the upper phase was discarded. The lower phase was counted using Aquasol. Protein was determined by the method of Lowry et al. (12).

Extraction of Lipids from Milk and Serum

Lipids were extracted by the method of Folch et al. (13). Milk and serum total lipids were transmethylated as described previously (14) and the fatty acid methyl esters analyzed by Hewlett Packard Gas Chromatograph using a 60-meter SP2340 glass capillary column.

Statistical Analysis

Student's *t*-test was used for statistical evaluation.

RESULTS

The body weights and the weights of hearts were decreased by 50% in the diet-restricted group. By day 21, the controls weighed 26.3 ± 2.6 g whereas the diet-restricted pups weighed only 13.4 ± 2.3 g ($P < 0.001$). The weights of the hearts were 0.17 ± 0.01 g and 0.07 ± 0.01 g for the controls and undernourished, respectively ($P < 0.001$). The size of the litter was

critical in the rate of β-oxidation. Only litters of identical size could be used. There were no significant changes in the fatty acid composition of serum or milk total lipids (Table 2). The total soluble proteins of the myocardium did not vary in the 2 groups.

Substrate concentration, protein dilution and cofactor requirements for the optimum β-oxidation rate for the heart were determined postnatally at 5, 14 and 21 days. β-Oxidation by the developing heart mitochondria was linear from 100-500 μg protein, differing from the values for the adult heart reported previously (5). The molar concentrations of carnitine, CoA and Mg^{++} required were similar to those reported for the adult heart (5). The optimal conditions were the same for 5, 14 or 21 days for both the control and the undernourished groups.

No significant difference was found in the β-oxidation of palmitate on day 5 in the heart homogenates of the control and undernourished pups (Fig. 1). The addition of carnitine did not alter the rate or shape of the curve. There was a 30% decrease in the production of $^{14}CO_2$ on day 14 ($P < 0.05$) and a 61% decrease on day 21 ($P < 0.001$) in the undernourished hearts compared with the controls. The addition of ATP did not enhance the oxidation. Further studies were restricted to day 21.

Table 3 shows the β-oxidation of heart mitochondria using [1-^{14}C]palmitate, [1-^{14}C]-palmitoyl CoA and [1-^{14}C]palmitoyl carnitine as substrates. With [1-^{14}C]palmitate, the rate of $^{14}CO_2$ produced was 2464 ± 317 cpm/mg protein/min for the control group and 1682 ± 91 for the diet-restricted group ($P < 0.002$). When [1-^{14}C]palmitoyl CoA or [1-^{14}C]-palmitoyl carnitine was the substrate, the oxidation rate of the heart mitochondria of the diet-restricted group was as high as in the control.

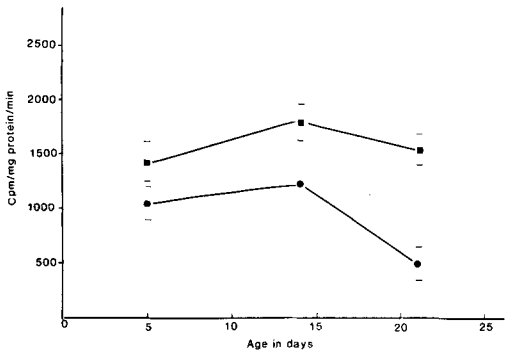

FIG. 1. β-Oxidation of developing heart homogenates. Values are mean ± S.D. from 3 experiments involving 9 pups from each group at each age. ■—■ Control; ●—● Diet-restricted.

Figure 2 gives the palmitoyl CoA synthetase activity of the heart homogenates and mitochondria. Unlike the case of the liver enzyme (11), additional EDTA was not required for optimum enzyme activity in developing heart homogenates. In fact, EDTA concentrations above 0.1 mM inhibited the enzyme activity by 40%. However, heart mitochondrial preparations contained 0.05 mM EDTA in both the control and experimental groups to prevent mitochondrial swelling (15). When the maternal diet was restricted after day 14 of pregnancy, even though the pups weighed significantly less than the controls, the palmitoyl CoA synthetase activity was not different in the 2 groups. When the diet was restricted at 7 days, the activity of the homogenates as well as mitochondria showed a significant decrease ($P < 0.001$).

DISCUSSION

Dietary fatty acid imbalances introduced pregestationally have been shown to influence the in vitro β-oxidation of fatty acids by heart

TABLE 2

Fatty Acid Composition of Total Lipids of Milk and Serum

Fatty acid	Milk[a]		Serum[b]	
	Control	Diet restricted	Control	Diet restricted
Saturates	51.2 ± 3.8	57.2 ± 1.2	37.0 ± 2.4	36.9 ± 1.4
Monoenes	21.5 ± 2.1	17.3 ± 0.9	13.2 ± 1.1	14.5 ± 2.0
Dienes	22.4 ± 1.3	20.1 ± 0.1	25.0 ± 1.4	22.8 ± 1.6
Tetraenes	1.9 ± 0.3	1.5 ± 0.2	16.6 ± 2.4	21.7 ± 3.5
Polyenes	0.7 ± 0.1	0.9 ± 0.1	5.0 ± 0.8	4.1 ± 0.5

Values are percent ± S.D.

[a]Gastric milk samples from 6 control and 7 diet-restricted pups at day 14 were used.

[b]Three rats at day 21 were used from each group.

TABLE 3
β-Oxidation of Heart Mitochondria from 21-Day-Old Rats

Substrate	Control cpm/mg protein/min	Diet restricted cpm/mg protein/min
[1-^{14}C] Palmitate	2463.9 ± 317.0	1681.6 ± 91.2[a]
[1-^{14}C] Palmitoyl CoA	3927.9 ± 974.3	3907.4 ± 1067.2
[1-^{14}C] Palmitoyl carnitine	4782.0 ± 771.8	4908.2 ± 1033.8

Values are mean ± S.D. from 3 experiments involving 9 pups from each group.
[a]$P < 0.002$.

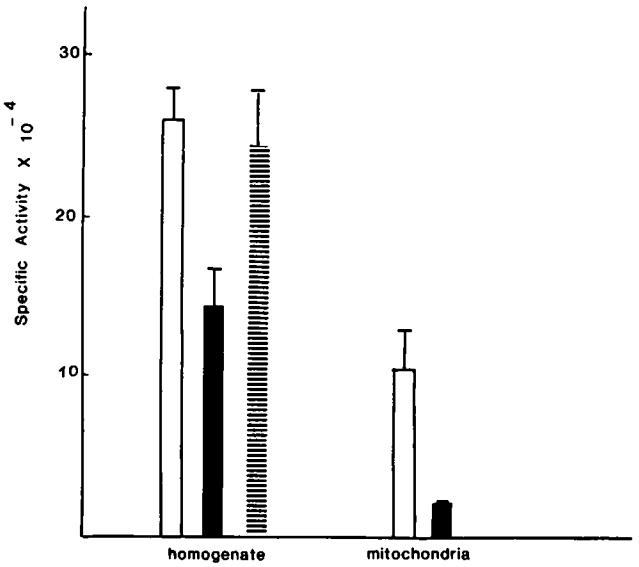

FIG. 2. Palmitoyl CoA synthetase activity in 21-day-old rat hearts. Specific activity, expressed as μ moles of [1-^{14}C] palmitate converted/mg protein/min, is the mean ± S.D. from 3 experiments involving 9 pups from each group. □ control; ■ diet restricted on 7th day of pregnancy; = diet restricted on 14th day of pregnancy.

homogenates of the progeny (5). In the present study, pregnant female rats were restricted to 50% of the ad libitum control intake whereas the number of young in each litter was equalized. At the level of 50%, the diet still provided adequate vitamins and minerals except vitamins B_6, E and K. Insufficient levels of pyridoxine and vitamin E are known to produce essential fatty acid deficiency in the rat (16,17). The role of vitamin K in lipid metabolism is unknown. Since in the present study no physical or biochemical evidence was found of essential fatty acid deficiency either in the dams or the pups (Table 2), a reasonable assumption would be that the deficiency introduced in the mothers is that of calories and is, in this sense, nonspecific in nature. There is, however, some evidence that the effect on the young stems more from protein deficiency than from calorie restriction (18).

In this technique, the assumption was made that the deficient diet influenced lactation quantitatively but not qualitatively (19). In the present study, we found that the β-oxidation of palmitate by the hearts of the undernourished pups decreased significantly during the suckling period. This significant finding led us to explore 4 main possibilities: an activation problem involving acyl thiokinase, inhibition of CoA carnitine transferase, low levels of carnitine and lack of sufficient ATP from the inhibition of energy-liberating processes. Even though carnitine was required by the β-oxidation system of the developing heart and carnitine concentration was found to be low at birth in the rat (20), increasing the concentration of carnitine in vitro did not alter the production of CO_2. Similarly, increasing ATP concentration also failed to enhance the production of CO_2 by the diet-restricted pups. Consequently, the possibility of an inhibition of activation or intra-

mitochondrial transport of fatty acids by heart mitochondria was studied in vitro by using [1-^{14}C]palmitate, [1-^{14}C]palmitoyl CoA and [1-^{14}C]palmitoyl carnitine. With [1-^{14}C]palmitate, a significant decrease ($P < 0.002$) in the production of $^{14}CO_2$ occurred, whereas with the other 2 substrates, the rates of β-oxidation were identical to control values, showing clearly that the activation of fatty acids was impaired. Further studies demonstrated that this inhibition was from a decrease in palmitoyl CoA synthetase activity (Fig. 2).

Long-chain fatty acyl CoA synthetase is localized in the outer membrane of mitochondria and microsomes (21,22). Unlike the liver, the adult heart showed no significant changes in carnitine palmitoyl transferase activity in rats (23) that had been fasting. In the developing heart, carnitine palmitoyl transferase activity was shown to be independent of dietary fat intake (24). The regulatory mechanisms involved in fatty acid oxidation may be quite different in the heart from those in the liver. Bailey and Lockwood had proposed that the formation of palmitoyl CoA seemed to be the rate-limiting step in palmitate oxidation (3). The present work also supports these findings and establishes the regulatory function of this enzyme in the β-oxidation of the developing heart. Recently, Olowe and Schulz (25) have shown that, in the presence of specific inhibitors, inactivation of the thiolase can cause the inhibition of fatty acid oxidation in rat heart mitochondria.

Why undernutrition causes the specific inhibition of this enzyme in the developing heart is not clear. In utero the fetus is exposed to a predominantly carbohydrate diet, which is replaced by a high-fat milk diet from birth to weaning. In the present study, the rate of β-oxidation is maintained at a high level throughout the suckling period in the control heart homogenates (Fig. 1). In the diet-restricted group, the oxidation increased from day 5 to 14 and then decreased, the level always being below the control value. The difference between the control and the deficient groups became progressively greater by the third week of suckling, tending to correlate the palmitoyl CoA synthetase activity with the fat content of the diet. Warshaw had suggested that the ability to oxidize fatty acids is influenced by the proportion of lipid in the diet (2). But if the maternal food intake is restricted from the last week of pregnancy, the palmitoyl CoA synthetase activity is unchanged from the control values, showing that other factors may also influence the induction of this enzyme in the developing heart.

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REFERENCES

1. Lockwood, E.A., and Bailey, E. (1970) Biochem. J. 120, 49-54.
2. Warshaw, J.B. (1972) Develop. Biol. 28, 537-544.
3. Bailey, E., and Lockwood, E.A. (1973) Enzyme 15, 239-253.
4. Barrie, S.E., and Harris, P. (1977) Am. J. Physiol. 233, H707-710.
5. Menon, N.K., and Dhopeshwarkar, G.A. (1983) Biochim. Biophys. Acta 751, 14-20.
6. Chow, B.F., and Lee, C.J. (1964) J. Nutr. 82, 10-18.
7. Kennedy, G.C. (1957) J. Endocrinol. 16, 9-17.
8. Control of Diets in Laboratory Animal Experimentation. From ILAR News, Vol. XXI, Number 2, Winter-Spring (1978), National Academy of Sciences, Washington, D.C.
9. Menon, N.K., Moore, C., and Dhopeshwarkar, G.A. (1981) J. Nutr. 111, 1602-1610.
10. Pande, S.V., and Blanchaer, M.C. (1971) J. Biol. Chem. 25, 402-411.
11. Bar-Tana, J., Rose, G., and Shapiro, B. (1975) in Enzymology (Lowenstein, J.M., ed.) Vol. XXXV, pp. 117-118, Academic Press, New York, NY.
12. Lowry, D.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
13. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497-509.
14. Menon, N.K., and Dhopeshwarkar, G.A. (1981) in Progress in Lipid Research (Holman, R.T., ed.) Vol. 20, pp. 129-134, Pergamon Press, England.
15. Van den Bergh, G.H. (1967) in Methods in Enzymology (Estabrook, R.W., and Pullman, M.E., eds.) Vol. X, p. 750, Academic Press, New York, NY.
16. Zehaluk, C.M., and Walker, B.L. (1973) J. Nutr. 103, 1548-1553.
17. Dhopeshwarkar, G., Subramanian, C., Gan-Elepano, M., and Mead, J.F. (1981) Abstract No. 782, XII International Congress of Nutrition, San Diego, CA.
18. Miller, S.A. (1969) in Mammalian Protein Metabolism (Munro, H.N., ed.) pp. 219-221, Academic Press, New York, NY.
19. Winick, M. (1976) in Malnutrition and Brain Development, p. 63, Oxford University Press, New York, NY.
20. Wittls, B., and Bressler, R. (1965) J. Clin. Invest. 44, 1639-1646.
21. Bressler, R. (1970) in Comprehensive Biochemistry (Florkin, M., and Stotz, E.H., eds.) Vol. 18, pp. 331-359, Elsevier, Amsterdam.
22. Hoppel, C.L., and Tomec, R.J. (1972) J. Biol. Chem. 247, 832-841.
23. Aaes, M., and Daae, N.W. (1971) Biochim. Biophys. Acta 239, 208-216.
24. Aprille, J.R. (1976) Pediatr. Res. 10, 982-985.
25. Olowe, Y., and Schulz, H. (1982) J. Biol. Chem. 257, 5408-5413.

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Effect of Antioxidants on Lipid Peroxidation in Iron-Loaded Rats

CORA J. DILLARD, JEANNE E. DOWNEY and **AL L. TAPPEL***, *Department of Food Science and Technology, University of California, Davis, CA 95616*

ABSTRACT

Indirect evidence has suggested that lipid peroxidation is associated with iron overload in vivo. As a measure of lipid peroxidation, pentane expired in the breath of rats loaded with an accumulated dose of either 100 mg or 186-200 mg of iron injected intraperitoneally as iron dextran was measured over a 7 to 8 week period, and the effect on pentane production of feeding antioxidant-supplemented diets was determined. By the seventh week of feeding the diets, rats fed 0.3% L-ascorbic acid produced 17% less ($P = 0.03$) pentane than did rats fed the basal antioxidant-deficient diet, whereas rats fed 0.004% dl-α-tocopherol acetate produced 92% less ($P < 0.001$). After being fed the basal diet for 7 weeks, iron-loaded rats produced 76 ± 9 pmol pentane/100 g body wt/min. When synthetic antioxidants were added to the diet at a concentration of 0.25%, the order of effectiveness in decreasing pentane production after 1 week was: N,N′-diphenyl-*p*-phenylenediamine > ethoxyquin > butylated hydroxyanisole > butylated hydroxytoluene > propyl gallate ≈ no antioxidant. After removal of either ethoxyquin or N,N′-diphenyl-*p*-phenylenediamine from the diets for 1 week, pentane production increased to a high level. The total amount of lipid soluble fluorophores in individual spleens of rats fed N,N′-diphenyl-*p*-phenylenediamine, ethoxyquin, dl-α-tocopherol acetate, ascorbic acid and no antioxidant were correlated significantly with the corresponding total integrated amount of pentane produced by the individual rats over the 7 to 8 week period. This study has provided some of the most direct evidence to date that lipid peroxidation is associated with iron overload in vivo.

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INTRODUCTION

Iron overload is a medical problem in a number of disorders commonly called hemochromatoses (1). Two causes of hemochromatoses are excessive iron absorption and parenteral iron overloading, each leading to an accumulation of iron in various body tissues. Jacobs (2) recently reviewed evidence that lipid peroxidation accompanies iron toxicity. Golberg and Smith (3,4) and Golberg et al. (5) were among the first researchers to undertake biochemical and histological investigations of the relationship of vitamin E and lipid peroxidation to iron overload in rats and mice. Rachmilewitz et al. (6) showed that the erythrocytes of thalassaemia major subjects with iron overload from repeated transfusions underwent excess lipid peroxidation that was associated with decreased levels of vitamin E. Heys and Dormandy (7) found spleens from humans with transfusional iron overload to peroxidize in vitro to form thiobarbituric acid reactants and lipid soluble fluorophores and also found vitamin E levels in spleens to correlate inversely with the degree of iron overload.

Pentane and ethane are volatile decomposition products of ω6- and ω3-unsaturated fatty acid hydroperoxides, respectively (8). These volatile products are exhaled in the breath of animals and can be used as indices of in vivo lipid peroxidation. The method of measuring pentane production by rats has been used to show that vitamin E (9) and N,N′-diphenyl-*p*-phenylenediamine (10), a synthetic antioxidant, effectively prevent in vivo lipid peroxidation. Elevated levels of pentane were exhaled by rats with a chronic iron overload (11). Dougherty et al. (12) showed that vitamin E, the most important biological free-radical scavenger, and selenium protected rats against lipid peroxidation induced by acutely toxic doses of ferrous chloride and iron dextran as measured by exhaled ethane (12). The potential usefulness of free-radical scavengers in the treatment of iron overload has been discussed (13). In the present study, the method of pentane measurement was used to evaluate the relative effectiveness of various antioxidant free-radical scavengers in the suppression of lipid peroxidation in iron-loaded rats initially fed a basal antioxidant-deficient diet. Additionally, chloroform-methanol extractable fluorescent lipid peroxidation products (14) were measured in the spleens of some of the groups of animals.

METHODS

Animals and Diets

Male Sprague-Dawley rats were obtained at 21 days of age from Simonsen Laboratories,

*To whom correspondence should be addressed.

Inc. (Gilroy, CA). The rats were fed, ad libitum, a basal diet (15) that contained 10% tocopherol-stripped corn oil and 20% casein (Teklad Test Diets, Madison, WI). Food was provided fresh daily or every second day.

Three experiments were carried out. Experiment I was a preliminary experiment to determine subsequent protocols and to determine the levels of pentane produced by rats fed the basal diet and injected intraperitoneally with saline in place of iron. One group of 6 rats was fed the basal antioxidant-deficient diet for 2 weeks before the first intraperitoneal injection of iron dextran (Burns Biotec, Omaha, NB). A second group of 4 rats was fed the basal diet that contained 0.004% dl-α-tocopherol acetate. During the third and fourth week of feeding the diet, each of the 10 rats was injected intraperitoneally with 100 mg of iron/100 g body weight to give a total of 200 mg of iron/100 g body weight. A third group of 4 rats was fed the basal diet, and each rat was injected twice with physiological saline in place of iron dextran.

Experiment II tested the ability of vitamin E and vitamin C (ascorbic acid) to inhibit lipid peroxidation in iron-loaded rats. The rats were divided into groups (n = number per group) and were fed the basal diet containing either (a) 0.004% dl-α-tocopherol acetate (n = 5), (b) 0.004% dl-α-tocopherol acetate and 0.3% L-ascorbic acid (Nutritional Biochemicals Corp., Cleveland, OH, n = 5), (c) 0.3% L-ascorbic acid (n = 7), (d) 0.1% L-ascorbic acid (n = 6) or (e) no addition (n = 7). Each rat was injected with 50, 50 and 100 mg of iron as iron dextran/100 g body weight on days 7, 14 and 21, respectively. These days corresponded to the beginning of weeks 2, 3 and 4 of the experiment. The final total dose was 186 ± 3 mg of iron/rat.

Experiment III tested the effectiveness of several synthetic antioxidants in decreasing lipid peroxidation in iron-loaded rats. Each rat was injected with a total of 200 mg of iron. The total dose of iron was given as 3 injections on days 2, 11 and 15 of the experiment. All rats were fed the basal antioxidant-deficient diet during the first 2 weeks and then they were divided into groups of 5 rats and fed (a) the basal diet with either 0.25% N,N′-diphenyl-*p*-phenylenediamine (DPPD, Goodrich Chemical Co., Cleveland, OH), (b) 0.25% ethoxyquin (a gift from Monsanto Co., St. Louis, MO), (c) 0.25% butylated hydroxytoluene (BHT, Eastman Chemical Products, Inc., Kingsport, TN), (d) 0.25% butylated hydroxyanisole (BHA, Eastman Chemical Products, Inc.), (e) 0.25% propyl gallate (Eastman Chemical Products, Inc.) or (f) no addition. The rats were fed the diets with BHA, BHT and propyl gallate only during the third, fourth and fifth weeks of the experiment. The rats fed ethoxyquin and DPPD were provided with antioxidant diets only during the third and fifth weeks. Each of 4 rats in another group was injected with only 100 mg of iron and was fed only the basal diet.

Gas Chromatographic Measurement of Pentane

In experiment I, pentane production was measured periodically from the fourth through the seventh week, and in experiment II, weekly for 7 weeks beginning 1 week after the individual diets were first fed to the rats. In experiment III, pentane production was measured weekly for 6 weeks beginning after 2 weeks of feeding the basal diet. The methods for collecting expired breath and the chromatographic analysis of short-chain hydrocarbons have been described previously (9). Rats that had fasted for approximately 18 hr were placed individually into a holding chamber supplied with hydrocarbon-free air (<0.01 ppm total hydrocarbons, Matheson, Newark, CA) for 15-20 min before 400-600 ml samples were collected. The time required to collect each sample was 4-5 min. The air-breath mixture leaving the head portion of the rat chamber passed through a small tube that contained a layer of 10-20 mesh Drierite (W. A. Hammond Drierite Co., Xenia, OH) and a layer of 20-30 mesh Ascarite II (A. H. Thomas Co., Philadelphia, PA) to remove water and carbon dioxide, respectively. The 10-ft alumina column was standardized daily with 0.89 ppm pentane in nitrogen (Matheson). The rates of pentane production were calculated as previously described (16).

Lipid Soluble Fluorescence

The rats in experiments II and III were killed at 12 weeks of age. They had been fed their various diets for 9 weeks. At this time, lipid soluble fluorescence in the spleen was measured in 1:20 tissue:solvent (chloroform/methanol, 2:1) extracts, according to the method described by Fletcher et al. (17). The excitation and emission spectra of each extract were recorded using a spectro-photofluorometer with a ratio attachment (American Instrument Co., Inc., Silver Spring, MD).

Statistical Analysis of Data

To determine the significance of differences between means of any 2 groups of data, the Student's *t* test was applied.

RESULTS

Pentane Production

In experiment I, the preliminary experiment, the rate of pentane produced by the antioxidant-deficient rats (n = 4) not injected with iron was 5 ± 1 pmol/100 g body wt/min after the basal diet had been fed for 7 weeks. Since these saline-injected control rats fed the basal diet produced low amounts of pentane, similar groups of animals were not included in experiments II and III. The rats that had been fed 0.004% dl-α-tocopherol acetate for 7 weeks (n = 4) and injected with a total of 200 mg of iron/100 g body wt produced 9 ± 1.8 pmol pentane/100 g body wt/min, and the rats that had been fed the basal diet for 7 weeks (n = 6) and injected with iron produced 69 ± 6 pmol/100 g body wt/min.

The results of experiment II are summarized in Figure 1, which shows the time course for the production of pentane by iron-loaded rats that were fed the basal diet with and without vitamin E and ascorbic acid. Whereas iron-loaded rats fed the vitamin E-supplemented diets exhaled pentane at low and essentially unchanged levels over the course of the experiment, whether or not ascorbic acid was included in the diet, the rats fed the basal diet exhaled levels of pentane that increased up to 17-fold over the initial levels. The maximum rate of pentane produced by the rats fed the basal diet was 58 ± 3.5 pmol/100 g body wt/min, while the maximum rate produced by the rats fed 0.004% dl-α-tocopherol acetate and no ascorbic acid was 5.5 ± 0.8 pmol/100 g body wt/min. At the fourth week and at each subsequent week, pentane production was higher ($p < 0.001$, 1-tailed test) in each of the 3 groups of rats fed the vitamin E-deficient diets than in the 2 groups of rats fed the vitamin E-supplemented diets. By the fourth week, the group of rats fed the basal diet with 0.3% ascorbic acid produced less ($p < 0.05$, 2-tailed test) pentane than did the groups fed the basal diet with and without 0.1% ascorbic acid. There were no significant differences in the weights of the various groups of rats by the end of the experiment and no deaths occurred.

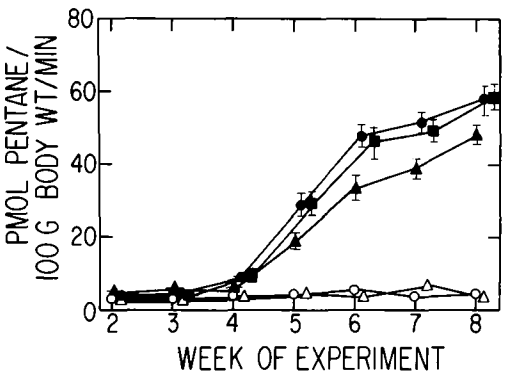

FIG. 1. The effect of dietary dl-α-tocopherol acetate and L-ascorbic acid on pentane production by iron-loaded rats. The basal diet contained no antioxidant (●), 0.1% L-ascorbic acid (■), 0.3% L-ascorbic acid (▲), 0.004% dl-α-tocopherol acetate (○) or 0.004% dl-α-tocopherol acetate and 0.3% L-ascorbic acid (△). A dose of 50, 50 and 100 mg of iron as iron dextran/100 g body wt was injected intraperitoneally into each rat on days 7, 14 and 21 (at the beginning of weeks 2, 3 and 4), respectively (186 ± 3 mg iron/rat). The standard errors for (○) and (△) were all ±0.6.

The results of experiment III are shown in Figure 2. Pentane production by the rats fed the basal diet and injected with a total of 100 mg of iron was measured only until the seventh week. At this time, the level of pentane production was 40 ± 5 pmol/100 g body wt/min compared with 76 ± 9 pmol/100 g body wt/min produced by the rats injected with a total of 200 mg of iron. The following results refer only to rats injected with 200 mg of iron. At the fourth week of the experiment, which was 1 week after the antioxidants were added to the diets, pentane production was less in the group of rats fed propyl gallate ($p < 0.02$, 2-tailed test) than by the rats fed the basal diet. Pentane production in the groups of rats fed BHA, BHT, DPPD and ethoxyquin was also lower (each $p < 0.001$, 2-tailed test) than in the rats fed the basal diet. This was the only time when rats fed propyl gallate produced less pentane than the rats fed the basal diet. The rats fed BHA produced less ($p = 0.03$, 2-tailed test) pentane than did the rats fed BHT, only after the antioxidants had been removed from the diets for 1 week (week 7, Fig. 2A). The dramatic result of adding DPPD or ethoxyquin to the diets is shown in Figure 2B. After feeding the rats with either of these 2 antioxidants as a supplement, pentane production was very low; however, the DPPD-fed rats produced significantly less pentane than the ethoxyquin-fed rats at all times. Two weeks after the removal of antioxidants from the diets, the pentane levels of the DPPD and BHA-fed groups were still lower ($p = 0.02$ and 0.005, respectively, 2-tailed test) than those of the rats fed the basal diet continuously. The other groups of rats had levels that were not significantly different from the group fed the basal diet. Overall, the order of effectiveness of these antioxidants, when fed for a short time period at a level of 0.25%, was DPPD > ethoxyquin > BHA > BHT > propyl gallate ≈ no antioxidant.

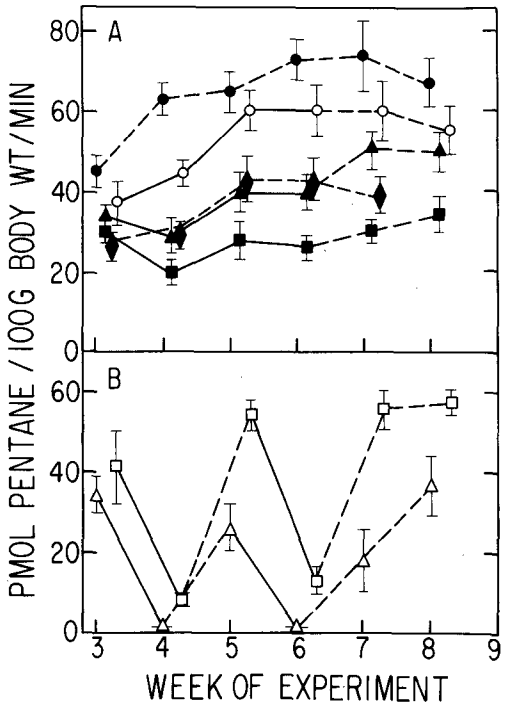

FIG. 2. The effect of antioxidants on pentane produced by rats injected with a total dose of 200 or 100 mg of iron as iron dextran. The dashed lines represent the times antioxidants were not fed and the solid lines represent the times when the antioxidant diets were fed. The antioxidant-deficient diet was also fed during weeks 1 and 2. The antioxidants fed were: (A) 0.25% BHT (▲), 0.25% BHA (■), 0.25% propyl gallate (○) and no antioxidant with a total dose of 200 mg of injected iron (●) and with a total dose of 100 mg of injected iron (◆); and (B) 0.25% ethoxyquin (□) and 0.25% DPPD (△).

No deaths occurred in the various groups while they were fed the antioxidant diets. When the initial weights of the animals were taken into account, there were no weight gain differences among the groups.

Lipid Soluble Fluorescence

Table 1 summarizes the data for the total amount of chloroform-methanol extractable fluorescent products in the spleens of some of the groups of rats. The spleens of the other groups were not analyzed. The maximum excitation and emission wavelengths varied only slightly among the groups. With the exception of the vitamin E-fed groups, the excitation maximum was 350-365 nm and the emission maximum was 440-450 nm. The excitation maximum of fluorophores from spleens of vitamin E-fed rats was in the range of 310-360 nm, a wider range than that for the other groups. In experiment II, the fluorescence of spleens from each of the 3 vitamin E-deficient groups of rats was higher than that from the 2 vitamin E-supplemented groups (all $p < 0.001$, 1-tailed test), and there was less ($p < 0.05$, 2-tailed test) fluorescence in the spleens of the group fed 0.1% ascorbic acid than in those fed the basal diet. The mean relative fluorescence value of the spleens from the group fed the 0.3% ascorbic acid diet was higher (162 ± 27) than that of the 0.1% ascorbic acid-fed group (104 ± 8), but the difference was not significant. In experiment III, the spleens of the rats injected with 200 mg of iron and fed the basal diet had significantly more fluorescence than did those of the groups fed DPPD ($p < 0.001$, 2-tailed test) or ethoxyquin ($p = 0.01$, 2-tailed test). These rats had been fed the antioxidant diets for only 12 days out of the 9 week experiment. The spleens of rats fed the basal diet and injected with 100 mg of iron had less ($p = 0.02$, 2-tailed test) fluorescence than those of rats fed the basal diet and injected with 200 mg of iron.

In experiments II and III, the total amount of pentane produced over the period during which measurements were made was calculated by integrating the values for all time points for each individual rat for which fluorophores were measured in the spleen. The linear regression correlation coefficients for a comparison of the total amount of pentane produced with the total amount of lipid soluble fluorophores in the spleen were $r = 0.61$ ($p < 0.001$) for experiment II and $r = 0.75$ ($p < 0.01$) for experiment III.

DISCUSSION

Jacobs (2) has reviewed the direct and indirect evidence that points to lipid peroxidation and membrane damage as a major factor in iron toxicity. This study has provided direct evidence that lipid peroxidation is involved by showing the effectiveness of vitamin E and some commercial synthetic antioxidants in the inhibition of iron-induced pentane production by rats; ascorbic acid was shown to be marginally effective. Iron overload in rats was an excellent model for the study of in vivo lipid peroxidation since sustained, high-level pentane production could be measured over a period of weeks when a diet with a high amount (~3.5%) of linoleic acid, an ω6-unsaturated fatty acid precursor of pentane, was fed to the rats. Iron-loading is easy to produce in rats by the intraperitoneal injection of iron dextran.

Over the past 20 years, numerous studies have been made of the ability of synthetic

TABLE 1

Effect of Dietary Antioxidants on Lipid Soluble Fluorophores in Spleens of Iron-loaded Rats

Dietary antioxidant	Excitation (nm)	Emission (nm)	Relative fluorescence[a] (total/spleen)	
Experiment II[b]				
0.004% dl-α-tocopherol acetate	310-315	425-450	14 ± 1	(4)[c]
0.004% dl-α-tocopherol acetate, 0.3% ascorbic acid	310-360	435-450	19 ± 2	(4)
antioxidant-deficient	355-365	440-445	150 ± 18	(5)
0.1% ascorbic acid	360	440-445	104 ± 8	(6)
0.3% ascorbic acid	355-360	440-445	162 ± 27	(7)
Experiment III[d]				
antioxidant-deficient[e]	355-360	445-450	66 ± 7	(5)
antioxidant-deficient[f]	350-360	440-450	38 ± 5	(4)
DPPD[d]	355-360	440-450	23 ± 3	(5)
ethoxyquin[d]	350-360	440-450	30 ± 8	(5)

[a]The fluorescence of a 0.2 g sample of each spleen was determined and the total fluorescence per spleen was calculated from the individual spleen weight. Values are expressed as mean ± S.E. relative to a standard of 1 μg of quinine sulfate/ml of 0.1 N H_2SO_4 that had a relative fluorescence of 500.

[b]Injected with a total dose of 186 mg iron as described in the text.

[c]Number in parentheses is number of animals used for fluorescence analysis. The rats used in both experiments were killed at 12 weeks of age and had been fed their respective diets for 9 weeks.

[d]The spleens of the other dietary groups of rats were not tested.

[e]Injected with a total dose of 200 mg of iron.

[f]Injected with a total dose of 100 mg of iron.

antioxidants to prevent various species-specific vitamin E-deficiency syndromes. As reviewed by Johnson (18), synthetic antioxidant replacement of vitamin E has met with limited success, perhaps because vitamin E has other important properties in addition to antioxidant properties. Although some other metabolic roles for vitamin E have been reported (19), the most studied role of vitamin E is as the major in vivo biological antioxidant. Synthetic antioxidants are required in the animal diet at much higher levels than vitamin E to effect equal protection, and they have been used at levels up to 1% (20) or 1.55% (21) of the diet. A level of 0.025% ethoxyquin or 88 IU/kg of vitamin E completely inhibited encephalomalacia in chicks (22), and a level of 0.025% DPPD, BHT or ethoxyquin delayed but did not prevent onset of dystrophy symptoms in guinea pigs (23). DPPD at 0.1% of the diet was sufficient to support reproduction in female rats when the diet contained 4% fat (24). Based upon these and numerous other literature reports of the in vivo effectiveness of synthetic antioxidants, and considering the 10% fat content of the diet used, the concentration of synthetic antioxidants selected to test as inhibitors of iron-induced lipid peroxidation was 0.25% of the diet. Ascorbic acid was used at 0.1 and 0.3% of the diet and dl-α-tocopherol acetate at 0.004% of the diet.

As expected from previous studies of oxidant-induced in vivo lipid peroxidation (16), vitamin E effectively suppressed pentane production. In iron-loaded rats fed the basal diet, pentane production was greatly elevated above the levels produced by normal rats, and increased over time as peroxidation increased and the rats were fed the antioxidant-deficient diet for a longer time. The excellent antioxidant effectiveness of DPPD and ethoxyquin compared with that of BHT, BHA and propyl gallate was shown by the cycling of pentane production from low levels when either DPPD or ethoxyquin was present in the diet to high levels when antioxidant was absent from the diet. Most synthetic antioxidants have relatively low biological activity because of rapid turnover and poor subcellular distribution (25). Vitamin E is a good antioxidant in vivo because of its superior qualities of absorption and retention (26). Also, vitamin E is largely associated with the membrane lipids (27) where peroxidation is most likely to occur, while ethoxyquin (27) and DPPD (25) are mainly distributed in the cytosol. Ethoxyquin is more rapidly absorbed, deposited in the tissues and lost from the tissues than is vitamin E (27). The rapid turnover in the tissues of DPPD and ethoxyquin was shown by the marked increase in pentane production when they were removed from the diets. The ranking of antioxidants according to

their in vitro effectiveness does not always correspond with the ranking by in vivo effectiveness since absorption, distribution and retention factors are involved in vivo. The overall ranking of the synthetic antioxidants in this study was DPPD > ethoxyquin > BHA > BHT > propyl gallate ≈ no antioxidant. Shull et al. (23) found that DPPD delayed the onset of vitamin E deficiency-related muscular dystrophy longer and more effectively than ethoxyquin.

That dl-α-tocopherol acetate, ascorbic acid, DPPD and ethoxyquin did lessen lipid peroxidation in vivo was also shown by the lower amounts of fluorescent products in the spleens of iron-loaded rats than in the spleens of rats not fed antioxidant. The effectiveness of DPPD and ethoxyquin was shown even though they were included in the diets for only 12 days out of the 8 or 9 weeks that the rats were fed the 10% corn-oil diet. The high significance of the correlation of spleen fluorophores with total pentane production confirms that the accumulated fluorophores in the iron-loaded rat spleen were directly related to the ongoing lipid peroxidation measured by pentane production. The inhibition by vitamin E and other antioxidants of buildup in tissues of lipid soluble fluorophores that are lipid peroxidation products has been shown previously in the rat (28) and the mouse (29). Also, Heys and Dormandy (7) found that the iron-overloaded spleens of thalassaemic subjects underwent further in vitro lipid peroxidation to produce similar lipid soluble fluorophores.

Ascorbic acid is required in the human diet although it is not required by rats. Ascorbic acid plays a dual role in oxidant-antioxidant reactions. It can serve an antioxidant function through a synergistic effect with phenolic-type antioxidants like vitamin E. Synergism is attributed to its ability to regenerate antioxidants by supplying hydrogen to the phenoxy radical (30) or its ability to function as an oxygen scavenger (31). Another reaction, however, is one that occurs when ascorbic acid participates in the propagation of free-radical lipid peroxidation reactions via the reduction of ferric iron to ferrous iron, a potent free-radical generator. In vitro, ascorbate releases iron from ferritin (32), a nonheme iron protein that is a major form of iron that accumulates in iron overload (2). Whether or not release occurs in vivo is still a matter of debate (33). Ferritin alone is inactive in promoting fatty acid oxidation, however, it has a marked effect when ascorbic acid is present (34,35). Ascorbic acid has been used clinically to increase iron excretion in response to desferrioxamine treatment (36) of thalassemic patients with iron overload, and some clinicians are concerned that high doses of ascorbate may precipitate lipid peroxidation in these patients (7,8). Chen (37) and Chen and Chang (38) have reported that excess vitamin C leads to erythrocyte hemolysis and increased thiobarbituric acid reactants in rat (37) and guinea pig tissues (38). The present study found evidence of less lipid peroxidation in rats given dietary ascorbic acid when no vitamin E was in the diet. This evidence included somewhat lower pentane production by rats fed 0.3% ascorbic acid and also a lower level of lipid soluble fluorescent product accumulation in the spleens of rats fed ascorbic acid. Whether or not ascorbic acid influenced the amount or form of iron in the spleen and other tissues is not known since the iron content was not analyzed.

In this study we have shown by measuring expired pentane that lipid peroxidation occurs in the rat loaded with iron. This was shown by testing the relative in vivo antioxidant effectiveness of biological and nonbiological antioxidants and the antioxidant synergist, ascorbic acid. Because peroxidation and membrane damage do occur in iron toxicity (2), this excellent model would also be applicable in testing the effect of other antioxidants and/or iron chelators. A potentially useful iron-chelating drug, 2,3-dihydroxybenzoic acid, that also can act as a free-radical scavenger (8), would be an interesting drug to test for the inhibition of iron-induced lipid peroxidation in vivo.

ACKNOWLEDGMENT

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REFERENCES

1. Powell, L.W., and Halliday, J.W. (1980) in Iron in Biochemistry and Medicine (Jacobs, A., and Worwood, M., eds.), Vol. II, pp. 461-498, Academic Press, New York, NY.
2. Jacobs, A. (1980) in Iron in Biochemistry and Medicine (Jacobs, A., and Worwood M., eds.), Vol. II, pp. 427-459, Academic Press, New York, NY.
3. Golberg, L., and Smith, J.P. (1958) Br. J. Exp. Pathol. 39, 59-73.
4. Golberg, L., and Smith, J.P. (1960) J. Pathol. Bacteriol. 80, 173-180.
5. Golberg, L., Martin, L.E., and Batchelor, A. (1962) Biochem. J. 83, 291-298.
6. Rachmilewitz, E.A., Lubin, B.H., and Shohet, S.B. (1976) Blood 47, 495-505.
7. Heys, A.D., and Dormandy, T.L. (1981) Clin. Sci. 60, 295-301.
8. Dumelin, E.E., and Tappel, A.L. (1977) Lipids 12, 894-900.

9. Dillard. C.J., Dumelin, E.E., and Tappel, A.L. (1977) Lipids 12, 109-114.
10. Downey, J.E., Irving, D.H., and Tappel, A.L. (1978) Lipids 13, 403-407.
11. Dillard, C.J., and Tappel, A.L. (1979) Lipids 14, 989-995.
12. Dougherty, J.J., Croft, W.A., and Hoekstra, W.G. (1981) J. Nutr. 111, 1784-1796.
13. Graziano, J.H. (1976) Birth Defects: Orig. Artic. Ser. 12, 135-143.
14. Tappel, A.L. (1975) in Pathobiology of Cell Membranes (Trump, B.F., and Arstila, A.U., eds.), Vol. 1, pp. 145-170, Academic Press, New York, NY.
15. Draper, H.H., Bergan, J.G., Chiu, M., and Csallany, A.S. (1964) J. Nutr. 84, 395-400.
16. Tappel, A.L., and Dillard, C.J. (1981) Fed. Proc. 40, 174-178.
17. Fletcher, B.L., Dillard, C.J., and Tappel, A.L. (1973) Anal. Biochem. 52, 1-9.
18. Johnson, F.C. (1979) CRC Crit. Rev. Food Sci. Nutr. 11, 217-309.
19. McCay, P.B., and King, M.M. (1980) in Vitamin E–A Comprehensive Treatise (Machlin, L.J., ed.), pp. 289-317, Marcel Dekker, Inc., New York, NY.
20. Harman, D. (1968) J. Gerontol. 23, 476-482.
21. Ames, S.R., Ludwig, M.I., Swanson, W.J., and Harris, P.L. (1956) Proc. Soc. Exp. Biol. Med. 93, 39-42.
22. Machlin, L.J., and Gordon, R.S. (1960) Proc. Soc. Exp. Biol. Med. 103, 659-663.
23. Shull, R.L., Ershoff, B.H., and Alfin-Slater, R.B. (1958) Proc. Soc. Exp. Biol. Med. 98, 364-366.
24. Draper, H.H., Goodyear, S., Barbee, K.D., and Johnson, B.C. (1958) Br. J. Nutr. 12, 89-97.
25. Csallany, A.S., and Draper, H.H. (1960) Proc. Soc. Exp. Biol. Med. 104, 739-742.
26. Witting, L.A. (1980) in Free Radicals in Biology (Pryor, W.A., ed.), Vol. 4, pp. 295-319, Academic Press, New York, NY.
27. Wiss, O., Bunnell, R.H., and Gloor, U. (1962) Vit. Horm. 20, 441-455.
28. Reddy, K., Fletcher, B., Tappel, A., and Tappel, A. (1973) J. Nutr. 103, 908-915.
29. Tappel, A., Fletcher, B., and Deamer, D. (1973) J. Gerontol. 28, 415-424.
30. Bauernfeind, J.C., and Pinkert, D.M. (1970) Adv. Food Res. 18, 219-315.
31. Cort, W.M. (1974) J. Am. Oil Chem. Soc. 51, 321-325.
32. Mazur, A., Baez, S., and Shorr, E. (1955) J. Biol. Chem. 213, 147-160.
33. Charlton, R.W., and Bothwell, T.H. (1976) Birth Defects: Orig. Artic. Ser. 12, 63-79.
34. Wills, E.D. (1966) Biochem. J. 99, 667-676.
35. Wills, E.D. (1972) Biochem. Pharmacol. 21, 239-247.
36. Hershko, C., Cook, J.D., and Finch, C.A. (1973) J. Lab. Clin. Med. 81, 876-886.
37. Chen, L.H. (1981) Am. J. Clin. Nutr. 34, 1036-1041.
38. Chen, L.H., and Chang, H.M. (1979), Int. J. Vitam. Nutr. Res. 50, 87-91.

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Inhibition of the Hormone-Sensitive Lipase in Adipose Tissue by Long-Chain Fatty Acyl Coenzyme A

DAVID L. SEVERSON* and **BRENDA HURLEY**, *Department of Pharmacology & Therapeutics, Faculty of Medicine, The University of Calgary, Calgary T2N 4N1, Canada*

ABSTRACT

The effects of free fatty acids and fatty acyl esters of coenzyme A and carnitine on the activity of a hormone-sensitive lipase preparation made from pigeon adipose tissue were determined. Oleic acid (100 μM) resulted in a 40% inhibition of lipase activity. A more potent inhibition of lipase activity was seen with long-chain fatty acyl CoA compounds. The concentration required for half-maximal inhibition with oleoyl CoA and palmitoyl CoA was 25-40 μM, whereas palmitoyl carnitine stimulated lipase activity. Activated lipase preparations (preincubated with Mg^{2+}, ATP, cyclic AMP and protein kinase) were 4-6 times more sensitive to inhibition by oleoyl CoA than were nonactivated preparations. An increase in cellular levels of fatty acyl coenzyme A could, therefore, contribute to the feedback inhibition of lipolysis in adipose tissue.

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INTRODUCTION

Free fatty acids (FFA) exert a negative feedback effect on adipose tissue by inhibiting rates of lipolysis (1). That hormones such as the catecholamines stimulate lipolysis by a cyclic AMP-dependent protein kinase-catalyzed phosphorylation and the activation of the hormone-sensitive lipase in adipose tissue (2-4) has been well established. The inhibitive effects of FFA on catecholamine-stimulated rates of lipolysis are associated with a reduction in the levels of cyclic AMP in rat (5) and human (6,7) fat cells, suggesting that the inhibition of adenylate cyclase by FFA may be the mechanism used by fatty acids to regulate lipolysis in adipose tissue.

The inhibition of lipolysis by FFA can be prevented by albumin, which seems to be caused by the binding of fatty acids (1). The presence of albumin in the fat-cell incubation medium also resulted in an increase in catecholamine-stimulated rates of lipolysis and in higher levels of cyclic AMP (6,8). However, albumin enhanced the lipolytic response to dibutyryl cyclic AMP (8). Therefore, fatty acids must also regulate some step(s) distal to the formation of cyclic AMP. Malgieri et al. (9) have reported that FFA inhibited the activity of an adipose tissue lipase preparation. The present investigations were initiated to examine in detail the effects of FFA and some fatty acid metabolites on the activity of a hormone-sensitive lipase preparation from adipose tissue.

MATERIALS AND METHODS

Hormone-sensitive lipase preparations were isolated as a pH 5.2 precipitate fraction from the abdominal adipose tissue of pigeons as described by Severson et al. (10). Lipase activity was measured with either ethanolic triolein or glycerol-dispersed triolein substrate preparations (10). Each assay contained triolein (glycerol tri[1-^{14}C]oleate, 125 μM), piperazine-N,N′-bis (2-ethane sulfonic acid) buffer (25 mM, pH 7) with defatted bovine albumin (0.05%, w/v), and either ethanol (1.25%, v/v) or glycerol (8.33%, v/v). Stock solutions of oleic acid (sodium salt) were prepared in water. Oleoyl CoA (potassium salt), palmitoyl CoA (free acid) and palmitoyl carnitine chloride were dissolved in 10 mM sodium acetate (pH 6). Lipase activity in the presence of FFA or the long-chain fatty acyl esters of CoA and carnitine was measured by adding various concentrations of the compounds to the triolein substrate emulsion; then assays were initiated by the addition of the lipase preparation. The radioactive fatty acid released during the assay incubation was measured as described previously (10). Incubations were performed in duplicate under conditions where assays were linear with respect to enzyme protein. A unit of lipase activity was arbitrarily defined as that amount of enzyme that catalyzed the release of 1 μmol of FFA (oleate) in 1 hr at 30 C.

Adipose tissue lipase preparations were activated by preincubation with $MgCl_2$ (5 mM), ATP (0.5 mM), cyclic AMP (0.01 mM), and protein kinase (beef heart, 100 μg/ml) for 5 min at 30 C in a total volume of 100 μl (10), followed by the addition of the radiolabeled triolein substrate emulsion containing the additions as indicated. Lipase activity was then measured as described above. Nonactivated lipase preparations were preincubations

*To whom correspondence should be addressed.

with $MgCl_2$ (5 mM) for 5 min before assay.

RESULTS

The effects of oleic acid and the long-chain fatty acyl esters of CoA and carnitine on lipase activity in pigeon adipose tissue are shown in Figure 1. Oleic acid (100 μM) resulted in a 40% inhibition of lipase activity. In a typical assay, less than 10% of the triolein substrate (125 μM) is hydrolyzed. Consequently, oleic acid would never accumulate to levels that could cause the inhibition of lipase activity in assays performed in the absence of an exogenous source of FFA. More pronounced inhibition was observed with palmitoyl CoA and oleoyl CoA, where concentrations of 25 and 40 μM, respectively, produced half-maximal inhibition (I_{50}). In contrast, the addition of palmitoyl carnitine resulted in the stimulation of lipase activity. A similar pattern of effects (I_{50} of 30 μM for fatty acyl CoA) was also observed when a lipase preparation from rat fat pads (Severson and Hurley, unpublished observations) was used.

Incubation of a lipase preparation for 30 min with an unlabeled triolein substrate emulsion and [1-^{14}C]oleoyl CoA (110,000 DPM), followed by extraction and thin-layer chromatographic separation of the lipids, indicated that no radioactivity was incorporated into triacylglycerols. Therefore, the inhibition of lipase activity by the long-chain fatty acyl coenzyme A compounds observed in Figure 1 is not due to the resynthesis of triolein during the assay incubation.

Since oleic acid is a product of the lipase reaction, standard assays are performed in the presence of defatted albumin (0.05% w/v or

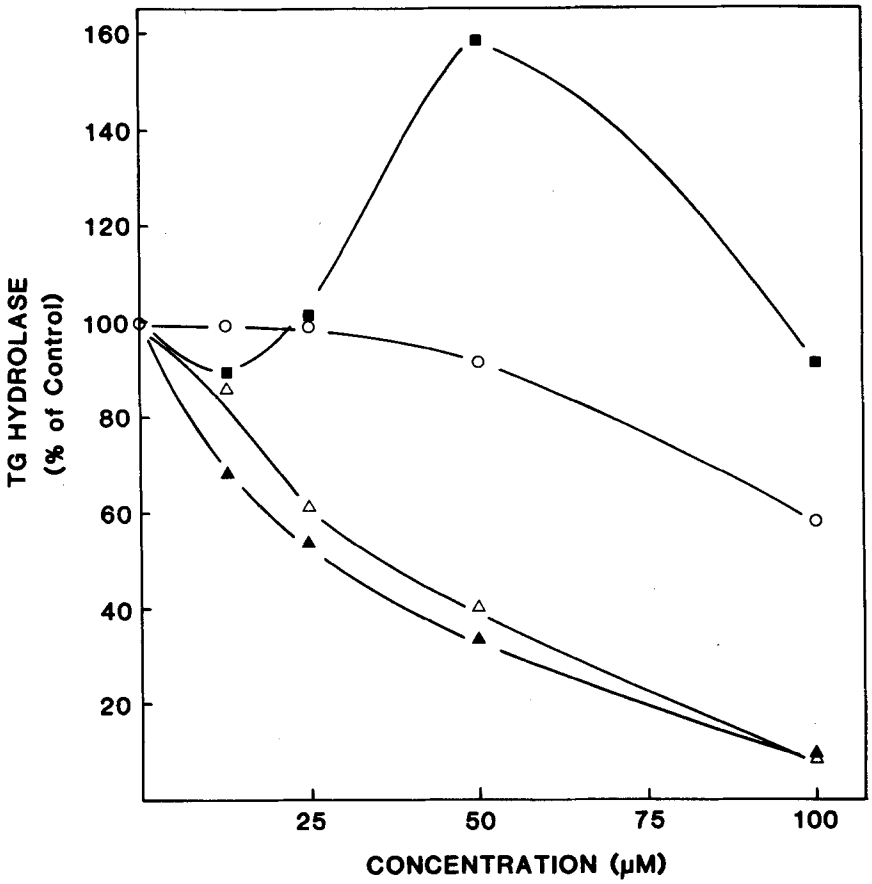

FIG. 1. Lipase activity in pigeon adipose tissue hormone-sensitive lipase preparations was measured with a glycerol-dispersed triolein substrate preparation in the presence of the indicated concentrations of oleic acid (○), oleoyl CoA (△), palmitoyl CoA (▲), or palmitoyl carnitine (■). The results are expressed as the percentage of control activity (100% = 1.44 units/mg protein) and are the mean of 2 experiments performed with different lipase preparations.

7.5 μM). Increasing the albumin content from 0.05% to 0.20% resulted in a reduction in the percentage of inhibition of lipase activity by 25 μM oleoyl CoA from 87% to 42% in assays with an ethanolic triolein substrate emulsion and 56% in assays with a glycerol-dispersed triolein substrate emulsion.

Preincubation of hormone-sensitive lipase preparations from pigeon adipose tissue with ATP, cyclic AMP and protein kinase has been shown to produce approximately a 4-fold increase in lipase activity (10). Therefore, the sensitivity of nonactivated and protein kinase-activated lipase preparations to inhibition by oleoyl CoA was investigated. As shown in Figure 2, protein kinase-activated lipase preparations were 4-6 times more sensitive to inhibition by oleoyl CoA (I_{50} values of 15 μM) when compared with the nonactivated lipase (I_{50} values of 55 μM for assays performed with glycerol-dispersed triolein and 100 μM for assays with ethanolic triolein substrate preparations). The I_{50} value of ca. 40 μM for oleoyl CoA presented in Figure 1 in assays performed with a glycerol-dispersed triolein substrate emulsion may be due to the presence of a small amount of activated lipase in the assay where no preincubation is done. The effect of oleoyl CoA was influenced by the substrate preparation since low concentrations of the fatty acyl CoA produced a stimulation of nonactivated lipase activity determined with an ethanolic triolein substrate emulsion (Fig. 2, panel A) that was not observed in assays with a glycerol-dispersed triolein substrate preparation (Fig. 1 and Fig. 2, panel B). The differential sensitivity to inhibition by long-chain fatty acyl CoA compounds was also observed with protein kinase-activated and nonactivated lipase preparations from chicken adipose tissue. Furthermore, the removal of metal ions and nucleotides from protein kinase-activated lipase preparations by gel exclusion chromatography on Biogel P6 columns (10) did not alter the increased sensitivity of the activated lipase to inhibition by palmitoyl CoA.

DISCUSSION

Previous studies by Malgieri et al. (9) have reported that lipase preparations from rat and chicken adipose tissue were inhibited at a FFA

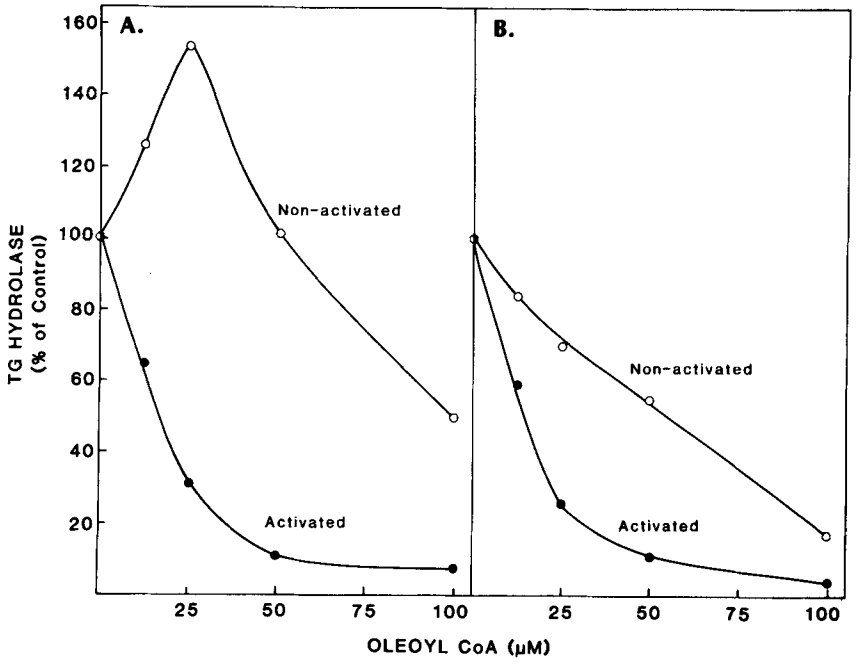

FIG. 2. Nonactivated (○) and activated (●) pigeon adipose tissue hormone-sensitive lipase preparations were assayed in the presence of the indicated concentrations of oleoyl CoA with either an ethanolic triolein (panel A) or a glycerol-dispersed triolein (panel B) substrate preparation. Results are expressed as the percentage of control activity and are the mean from experiments with 2 different lipase preparations. Absolute activities corresponding to the 100% value for the nonactivated and activated lipase were 1.80 and 7.93, respectively, with the ethanolic triolein substrate (panel A), and were 1.45 and 5.36, respectively, with the glycerol-dispersed triolein substrate (panel B).

(oleate)/albumin ratio of 3:9. In the present investigation, a 40% inhibition of both pigeon and rat adipose tissue lipases were observed with 100 μM oleic acid. Since the standard assay incubation also contained 7.5 μM albumin, this result corresponds to a FFA/albumin ratio greater than 10. This discrepancy in the sensitivity of the lipase preparations of adipose tissue to inhibition by FFA may be caused by differences in experimental conditions since the assay described by Malgieri et al. (9) contained 10 times more triolein and 50 times more albumin than the assay (10) used in this investigation.

A more potent inhibition of lipase activity (I_{50} values of 25-40 μM) was observed in experiments using long-chain fatty acyl CoA compounds. This inhibition could be reduced by increasing the albumin content of the assay. Therefore, the presence of albumin determines not only the absolute specific activity of the lipase but also the relative sensitivity toward the inhibition by fatty acyl CoA compounds. Concentrations of long-chain fatty acyl CoA in fat cells have been reported to range from 90 to 160 μM (11,12), although a portion of it will undoubtedly be bound to intracellular proteins.

The physiological significance of any observed inhibition of enzyme activities by FFA and fatty acyl CoA is controversial as these inhibitions could be the consequence of a nonspecific inactivation of the enzyme because of the detergent properties of the compounds (13,14). However, a number of recent investigations have presented evidence in favor of a physiological role in metabolic regulation for fatty acyl CoA compounds. First, an analog of oleoyl CoA has been shown to have similar detergent properties to the parent compound, but the analog produced much less inhibition of citrate synthase (15). Second, site-specific binding of fatty acyl CoA has been reported with citrate synthase (16), acetyl CoA carboxylase (17) and glucokinase (18,19). Third, the inhibition of glucokinase by fatty acyl CoA has been shown to be instantaneous and reversible (18). Finally, a reexamination of the critical micelle concentration for palmitoyl CoA has given a value of 30-60 μM, which is more than an order of magnitude greater than previously reported values (20). This finding suggests that the inhibition of enzymes by concentrations of fatty acyl CoA of less than 30 μM may not be due to the formation of micelles and the resultant detergent action, but rather to represent a physiologically significant form of metabolic regulation. Any demonstration of specificity in the inhibition of enzymes by fatty acyl CoA also implies a physiological significance. The activation of lipase preparations of adipose tissue by the cyclic AMP-dependent protein kinase resulted in an enhanced sensitivity to inhibition by oleoyl CoA, thus arguing for a specific and selective action of the fatty acyl CoA on the lipase. Palmitoyl carnitine, an acyl ester with detergent properties (21), did not inhibit lipase activity. Therefore, the inhibition of the adipose tissue lipase by long-chain fatty acyl CoA may contribute to the regulation of lipolysis. The presence of excess FFA would be expected to increase intracellular levels of fatty acyl CoA as a consequence of the reaction catalyzed by fatty acyl CoA synthetase. The selective inhibition of the protein kinase-activated form of the lipase by fatty acyl CoA, coupled with a decrease in the formation of cyclic AMP because of FFA (5-7), would constitute a very effective feedback control mechanism for lipolysis. Lipolytic hormones also increase the rates of reesterification (22), so presumably the fatty acyl CoA is then diverted to triacylglycerol synthesis. Long-chain fatty acyl CoA has also been shown to inhibit acetyl CoA carboxylase (23). This inhibition contributes to the regulation of the production of FFA by the lipogenesis pathway. Acetyl CoA carboxylase is also regulated by covalent modification (24). Note that the phosphorylated (inactive) form of hepatic acetyl CoA carboxylase is more sensitive to inhibition by fatty acyl CoA than is the dephosphorylated form (25,26).

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REFERENCES

1. Rodbell, M. (1965) Ann. N.Y. Acad. Sci. 131, 302-314.
2. Steinberg, D. (1976) Adv. Cyclic Nucleotide Res. 7, 157-198.
3. Severson, D.L. (1979) Can. J. Physiol. Pharmacol. 57, 923-937.
4. Fredrikson, G., Stralfors, P., Nilsson, N.O., and Belfrage, P. (1981) J. Biol. Chem. 256, 6311-6320.
5. Fain, J.N., and Shepherd, R.E. (1975) J. Biol. Chem. 250, 6586-6592.
6. Burns, T.W., Langley, P.E., and Robison, G.A. (1975) Metabolism 24, 265-276.
7. Burns, T.W., Langley, P.E., Terry, B.E., and Robison, G.A. (1978) Metabolism 27, 1755-1762.
8. Allen, D.O. (1979) Biochem. Pharmacol. 28, 733-736.
9. Malgieri, J.A., Shepherd, R.E., and Fain, J.N. (1975) J. Biol. Chem. 250, 6593-6598.

10. Severson, D.L., Fletcher, T. Groves, G., Hurley, B., and Sloan, S. (1981) Can. J. Biochem. 59, 418-429.
11. Denton, R.M., and Halperin, M.L. (1968) Biochem. J. 110, 27-38.
12. Saggerson, E.D., and Greenbaum, A.L. (1970) Biochem. J. 119, 193-219.
13. Pande, S.V., and Mead, J.F. (1968) J. Biol. Chem. 243, 6180-6185.
14. Taketa, K., and Pogell, B.M. (1966) J. Biol. Chem. 241, 720-726.
15. Hsu, K.-H. L., and Powell, G.L. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 4729-4733.
16. Caggianno, A.V., and Powell, G.L. (1979) J. Biol. Chem. 254, 2800-2806.
17. Ogiwara, H., Tanabe, T., Nikawa, J., and Numa, S. (1978) Eur. J. Biochem. 89, 33-41.
18. Tippett, P.S., and Neet, K.E. (1982) J. Biol. Chem. 257, 12839-12845.
19. Tippett, P.S., and Neet, K.E. (1982) J. Biol. Chem. 257, 12846-12852.
20. Powell, G.L., Grothusen, J.R., Zimmerman, J.K., Evans, C.A., and Fish, W.W. (1981) J. Biol. Chem. 256, 12740-12747.
21. Adams, R.J., Cohen, D.W., Gupte, S., Johnson, J.D., Wallick, E.T., Wang, T., and Schwartz, A. (1979) J. Biol. Chem. 254, 12404-12410.
22. Vaughan, M., and Steinberg, D. (1963) J. Lipid Res. 4, 193-199.
23. Halestrap, A.P., and Denton, R.M. (1973) Biochem. J. 132, 509-517.
24. Brownsey, R.W., Hughes, W.A., and Denton, R.M. (1979) Biochem. J. 184, 23-32.
25. Carlson, C.A., and Kim, K.-H. (1974) Arch. Biochem. Biophys. 164, 490-501.
26. Witters, L.A., Moriarity, D., and Martin, D.B. (1979) J. Biol. Chem. 254, 6644-6649.

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Plasma Triacylglycerol Turnover in Rats Using Labeled Glycerol

LETTER TO THE EDITOR

Sir:

The synthesis and turnover of circulating very low density lipoprotein-triglycerides (VLDL - TG) in rats under various physiological conditions have been studied by many investigators (1-3). Unfortunately, several papers have appeared in the last 8 years that fail to recognize that some of the tracer techniques and assumptions that have been applied to human subjects (4-6) are not applicable to rats (2,3,7, 8). I do not intend to belittle or detract from the interesting experiments that have been performed in these recent studies (9-11), especially since the aim in each case was to compare rates of plasma VLDL-TG turnover in two or more experimental conditions (rather than to establish some absolute value of plasma TG synthesis and/or removal). However, the possibility exists that these approaches might be applied incorrectly by other workers who have a primary interest in hepatic and intestinal secretory rates or in the rates of VLDL-TG removal from the circulation. Moreover, unresolved quantitative inconsistencies (both internal and in relation to the existing literature) in some of these papers can be readily understood, provided that earlier, validated analytical approaches are applied to the kinetic data. My position is that if one wishes to compare rates of plasma TG turnover in two conditions, using indices of liver TG turnover rates for these comparisons is not scientifically valid; yet, that is what, in my opinion, some investigators have been doing in a few (not all) of their studies. To their credit, I would like to emphasize that, in several instances, the authors also used additional experimental approaches (such as reinjection of labeled plasma TG or Triton WR-1339) to test their hypotheses.

Our interest, then, is clearly different from that of the original investigators. We wish to be able to review a body of published literature based upon various experimental approaches so that we may compare the rates of synthesis, turnover and use of circulating metabolites. For me, the best approximations of the "true" rates (at present, no method, tracer or non-tracer, that we know of, can be claimed to measure absolute rates of plasma VLDL-TG secretion in vivo) that one can calculate should be generated from this existing data base. In the studies of Cenedella et al. (9), an approach later applied by two other groups (10,11), the information that theoretically should provide a good approximation of plasma VLDL-TG turnover has not been used for that purpose.

I shall try to clarify this point, using the first of these papers (9) as an example. The approach of Cenedella et al. is based on a model validated by Farquhar et al. (4) in human subjects and later applied by Nikkila and Kekki (6) to whom the authors (9) refer. Farquhar et al. make the important point that their model does not apply to rats, rabbits or dogs. One may add that the model used in the early human studies (4-6) does not apply to any other species, including mice (12) and pigs (13), except, perhaps, chickens (14). The experimental approach involves the intravenous injection of [2-^{3}H]glycerol into the animal followed by the measurement of plasma- (or VLDL-) TG radioactivity and mass as a function of time. Typically, in all species studied, a delay is followed by the rapid appearance of label in plasma TG. The radioactivity reaches a maximum, the time and height of which vary in different species and conditions. Then the curve falls relatively slowly.

Farquhar et al. showed experimentally that this slow fall (after t_{max}) reflects the fractional turnover rate of plasma VLDL-TG in human subjects (4) (see Fig. 1B). This result agreed with the earlier finding of Friedberg et al. (15), and the technique has subsequently been used in humans by various investigators because of its apparent great simplicity (5,6,16). More sophisticated approaches, that take into account plasma glycerol (or FFA) turnover, complexities of hepatic TG turnover, recycling phenomena, and delipidation delays during plasma VLDL-TG degradation, have now been developed to measure plasma VLDL-TG turnover in human subjects (17-18). In no other experimental animal studied so far does the falling portion of the VLDL-TG curve after injection of labeled glycerol or FFA reflect the turnover of plasma VLDL-TG. Rather, as shown by Baker and Schotz (3) in rats, by Havel et al. in rabbits (19), by Gross et al. in dogs (8), and by Hannan et al. in pigs (13), this slope is determined to a major extent by the turnover of some of the liver's more rapidly turning over TG compartments, including TG in

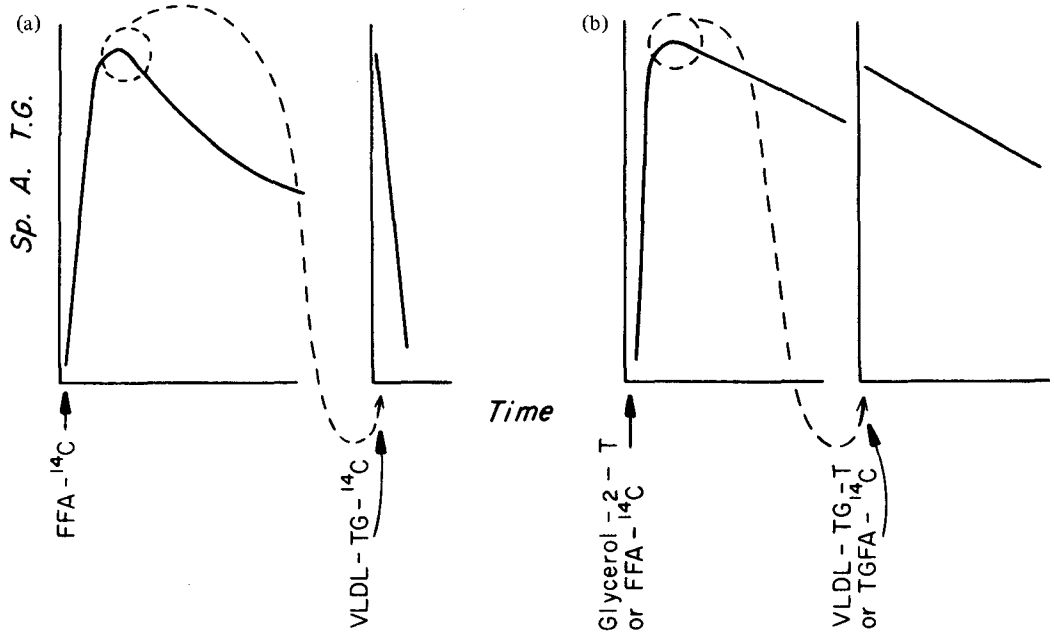

FIG. 1. Diagrammatic illustration of differences in the kinetic behavior of biologically labeled plasma VLDL-TG following intravenous injection of tracer in (A) rats and (B) human subjects. In *A*, the left panel shows the kinetic behavior of labeled plasma VLDL-TG after iv injection of ^{14}C-labeled palmitate complexed to serum albumin. In the right-hand panel of *A*, the kinetic behavior of labeled plasma VLDL-TG is shown following iv injection of tracer VLDL-TG that has been collected from donor rats at the time of maximum TGFA sp.act. (left panel). The falling slope of the curve in the right panel (A) reflects the rising, not the falling, slope of the curve shown in the left panel (A). When the same type of experiment is done in human subjects (B), the VLDL-TG curve (right panel) closely reflects the falling, not the rising, slope, of the curve shown in the left panel.

rough and smooth endoplasmic reticulum and Golgi apparatus (20). The plasma VLDL-TG compartment, which has a more rapid turnover, has only a minor influence on the falling portion of the curve, especially in rats. The early rising part of the curve should represent primarily the fractional rate constant of plasma VLDL-TG turnover provided the latter is faster than its precursor, which is almost certainly the case in rats (2,3), rabbits (19), dogs (8) and pigs (13) (see Fig. 1A). The essential features of the hepatic-(or intestinal) TG and plasma VLDL-TG system, when treated as a simple 2-compartment model (with a delay), are most clearly presented in the paper by Farquhar et al. (4) and by Gross et al. (8). In the former study, which is concerned with human subjects, the rationale for using the falling part of the VLDL-TG curve to represent the fractional turnover rate of plasma VLDL-TG directly is carefully developed. The authors (4) argue that since the plasma VLDL-TG sp.act. curve after injection of labeled FFA or glycerol falls (post-t_{max}) at the same rate as labeled VLDL-TG (reinjected), the liver TG precursor compartment must turn over faster than plasma VLDL-TG. In that case, the rising part of the plasma VLDL-TG curve would be primarily a reflection of the hepatic TG precursor compartment's fractional turnover—a situation that may be unique to human subjects.

In the paper by Gross et al. (8), an analysis of plasma VLDL-TG synthesis and turnover in dogs shows clearly that the approach of either Farquhar et al. (4) or of Cenedella et al. (9) would yield erroneously slow fractional turnover rates of plasma VLDL-TG turnover by a factor of 3. That is, the VLDL-TG sp.act. curve declined at a fractional rate of 0.48 hr^{-1}, the same rate as for the liver TG compartment, after rising at a fractional rate of 1.6 hr^{-1}, which is 3 times faster than the hepatic TG precursor compartment. This result was confirmed by reinjecting labeled VLDL-TG, which turned over at the rates predicted by the *rising* phase of the sp.act. curve of VLDL-TG after an injection of labeled glycerol. The logic used by Gross et al. is the same as that used in the multicompartmental analysis of Baker and Schotz (3), where the initial estimates in the case of rats were made on the basis of a simple 2-compartment model such as that used earlier

by Baker et al. (21) to analyze another system. In pigs, the liver TG (precursor) compartment turns over with a measured $t_{1/2}$ of 2 hr. This rate is also reflected in the slope of the falling limb of the VLDL-TG curve after a single injection of [2-^{3}H]glycerol. On the other hand, the VLDL-TG curve rises with a $t_{1/2}$ of 10 min, the mean value found experimentally for the fractional turnover rate of VLDL-TG when reinjected into recipient pigs (13). Thus, as Farquhar et al. pointed out (4), the declining slope of the sp.act. curve of VLDL-TG after the injection of labeled FFA or glycerol reflects the fractional turnover rate of plasma VLDL-TG in only one condition, namely, when the liver precursor TG pool turns over faster than the plasma VLDL-TG compartment. This is not the case in rats (3).

If I am correct, then rates of plasma VLDL-TG turnover, measured after the injection of tracer glycerol, have been grossly underestimated in several studies (9-11). However, when the published data in references 9-11 are reanalyzed according to the model of Baker and Schotz (3) or of Gross et al. (8), the rates of VLDL-TG turnover become consistent both with otherwise inconsistent data presented by the authors (9) and with most of the other extensive literature on VLDL-TG turnover in rats (Bird, Williams, and Baker, manuscript submitted).

NOME BAKER
Crump Institute of Medical Engineering
6417 Boelter Hall
University of California at Los Angeles
Department of Medicine
UCLA School of Medicine
Los Angeles, California 90024

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REFERENCES

1. Baker, N. (1982) in Lipoprotein Kinetics and Modeling (Berman, M., and Phair R.D., eds.) pp. 253-269 Academic Press, New York.
2. Laurell, S. (1959) Acta Physiol. Scand. 47, 218-232.
3. Baker, N., and Schotz, M.C. (1964) J. Lipid Res. 5, 188-197.
4. Farquhar, J.W., Gross, R.C., Wagner, R.M., and Reaven, G.M. (1965) J. Lipid Res. 6, 119-134.
5. Reaven, G.M., Hill, O.B., Gross, R.C., and Farquhar, J.W. (1965) J. Clin. Invest. 44, 1826-1833.
6. Nikkila, E.A., and Kekki, M. (1971) Acta Med. Scand. 190, 49-59.
7. Schotz, M.C., Baker, N., and Chavez, N.M. (1964) J. Lipid Res. 5, 569-577.
8. Gross, R.C., Eigenbrodt, E.H., and Farquhar, J.W. (1967) J. Lipid Res. 8, 114-125.
9. Cenedella, R.J., Crouthamel, W.C., and Mengoli, H.F. (1974) Lipids 9, 35-42.
10. Huang, M.-T., and Williams, M.A. (1980) Am. J. Physiol. 238, E499-E505.
11. Krause, B., Dory, L., and Roheim, P.S. (1982) Biochim. Biophys. Acta 710, 471-476.
12. Salmon, D.M.W., and Hems, D.A. (1973) Biochem. J. 136, 551-563.
13. Hannan, S.F., Baker, N., Rostami, H., Lewis, K., and Scott, P.J. (1980) Atherosclerosis 37, 55-68.
14. Kudzma, D.J., St. Claire, F., DeLallo, L., and Friedberg, S.J. (1975) J. Lipid Res. 16, 123-133.
15. Friedberg, S.J., Klein, F.R., Trout, D.L., Bogdonoff, M.D., and Estes, E.H., Jr. (1961) J. Clin. Invest. 40, 1846-1855.
16. Olefsky, J., Farquhar, J.W., and Reaven, G.M. (1974) Eur. J. Clin. Invest. 4, 121-127.
17. Zech, L.A., Grundy, S.M., Steinberg, D., and Berman, M. (1979) J. Clin. Invest. 63, 1262-1273.
18. Grundy, S.M., Mok, H.Y.I., Zech, L., Steinberg, D., and Berman, M. (1979) J. Clin. Invest. 63, 1274-1283.
19. Havel, R.J., Felts, J.M., and Van Duyne, C.M. (1962) J. Lipid Res. 3, 297-308.
20. Stein, Y., and Shapiro, B. (1959) Am. J. Physiol. 196, 1238-1241.
21. Baker, N., Shreeve, W.W., Shipley, R.A., Incefy, G.E., and Miller, M. (1954) J. Biol. Chem. 211, 575-592.

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METHODS

Analysis of Triglyceride Species by High-Performance Liquid Chromatography Via a Flame Ionization Detector

F.C. PHILLIPS[a], W.L. ERDAHL[a], J.D. NADENICEK[b], L.J. NUTTER[b], J.A. SCHMIT[c], and O.S. PRIVETT[a,*] [a]*The Hormel Institute, University of Minnesota, 801 16th Avenue N.E., Austin, MN 55912;* [b]*Nu-Chek-Prep, Inc., P.O. Box 172, Elysian, MN 56028; and* [c]*E. I. DuPont de Nemours & Co., Wilmington, DE 19898*

ABSTRACT

The analysis of triglyceride species by high performance liquid chromatography (HPLC) with a flame ionization detector (FID) and reversed-phase chromatography using chemically bonded octadecyl silane (ODS) Zorbax columns and gradient or isocratic solvent elution with methylene chloride/acetonitrile is described. Triglycerides containing acyl groups of critical pairs, *trans* and positional isomers, as well as mixtures of even and odd chain lengths are separated. Identification of triglycerides is made on the basis of retention times compared with equivalent and theoretical carbon numbers, and comparison with chromatograms of reference triglyceride mixtures. The methodology is demonstrated by fractionizing the triglycerides of olive oil under different chromatographic conditions using single and coupled conventional 250 × 4.6 mm columns and a short 80 × 6.2 mm column for fast separations.

Lipids 19:142-150, 1984.

INTRODUCTION

The application of reversed-phase liquid chromatography to glyceride species was demonstrated by Nickell and Privett (1) with the separation of triglycerides differing by one methylene group, one double bond or a *trans* unsaturated acyl group by a gravity flow procedure in which fractions were collected for analysis by gas liquid chromatography (GLC). When continuous detectors of the refractive index type became available for liquid chromatography, Pei et al. (2) developed a fast, simple procedure for the separation of simple saturated triglycerides by modern high performance liquid chromatography (HPLC) using a reversed-phase system of chemically bonded octadecyl silane (ODS)-silica as the stationary phase.

In an extension of this technique, Plattner and colleagues (3,4) demonstrated the separation of triglyceride species on the basis of chain length and unsaturation and obtained baseline separation of critical pairs of triglycerides containing oleic and palmitic acid by the addition of silver nitrate to the solvent system. These investigators suggested the separation of triolein and tripalmitin as a guide for determining column efficiency and estimated that ODS columns of ca. 15,000 plates should be adequate for the separation of the triglyceride species of these acids other than isomers, which require enzymatic techniques for an analysis. El-Hamdy and Perkins (5) and Perkins et al. (6) obtained similar types of separations directly with ODS columns. They developed the concept of theoretical carbon number (TCN) to define species separation of unsaturated triglycerides. The TCN is a very useful concept for the identification of unsaturated triglyceride species and assists in identifying triglycerides in this work.

In the studies by El-Hamdy and Perkins (5), standard 5 μ ODS columns of ca. 250 × 4.6 mm were used. These columns and the solvent systems employed were geared to an elution time of ca. 30 min. Recently Dong and DiCesare (7) demonstrated that similar separations could be made in about one-half the time with shorter (100 × 4.6 mm) columns packed with 3 μ ODS particles. In the abovementioned studies, a refractive index or UV detector with isocratic elution was used. In earlier work (8-12), we demonstrated the separation of the lipid classes by HPLC using gradient elution systems with a flame ionization detector (FID). In the present study, the separation of reference mixtures of triglycerides by HPLC using reversed-phase chromatography with ODS columns and gradient or isocratic elution is demonstrated and applied to olive oil under different chromatographic conditions.

MATERIALS AND METHODS

Reference Triglyceride Mixtures

The following reference mixtures of highly purified >99% triglycerides were obtained from Nu-Chek Prep, Inc., Elysian, MN.

HPLC #G-1. This mixture consists of the following pure glycerol triesters in the ratio 3:2:1 (even glyceride carbon #:odd glyceride carbon

*To whom correspondence should be addressed.

#:unsaturated glyceride): tricaprylin (24:0), trinonanoin (27:0), tricaprin (30:0), triundecanoin (33:0), trilaurin (36:0), tritridecanoin (39:0), trimyristin (42:0), tripentadecanoin (45:0), tripalmitin (48:0), tripalmitolein (43:3Δ9), trihepatadecanoin (51:0), tristearin (54:0), triolein (54:3Δ9), trilinolein (54:6Δ9,12) and trilinolenin (54:9Δ9,12,15).

HPLC #G-3. A mixture of triolein (54:3Δ9) and tripetroselinin (54:3Δ6) of equal weight.

HPLC Interesterified Test Mixtures. These mixtures consist of the following triglyceride molecular species.

Mixture A: $P_3 - P_2O_1 - P_1O_2 - O_3$ where P = palmitate and O = oleate.

Mixture B: $P_3 - P_2Pa_1 - P_1Pa_2 - Pa_3$ where P = palmitate and Pa = palmitoleate.

Mixture C: $La_3 - La_2Ln_1 - La_1Ln_2 - Ln_3$ where La = laurate and Ln = linolenate.

Mixture D: $M_3 - M_2L_1 - M_1L_2 - L_3$ where M = myristate and L = linoleate.

Mixture E: $P_3 - P_2H_1 - P_1H_2 - H_3$ where P = palmitate and H = heptadecanoate.

Mixture F: $P_3 - P_2O_1 - P_1O_2 - O_3 - P_2L_1 - POL - O_2L_1 - P_1L_2 - O_1L_2 - L_3$ where P = palmitate, O = oleate and L = linoleate.

Mixture G: $O_3 - O_2E_1 - O_1E_2 - E_3$ where O = oleate (18:1 Δ9-*cis*) and E = elaidate (18:1 Δ9-*trans*).

Pure olive oil was obtained from Dr. Eduardo Vioque of the Institute de La Grasa Sus Derivados (C.S.I.C.), Sevilla, Spain.

HPLC

HPLC was carried out with a Spectra Physics Model 3500 B liquid chromatograph equipped with a FID of our own design (13). Three ODS Zorbax columns, obtained from E. I. Dupont De Nemours and Company, were used in this work. Columns I and II were 250 x 4.6 mm and varied in their porosity and carbon content, the first having ca. 15% and the second ca. 6%. The third column (III) was a Golden Series Zorbax ODS column, 60 x 6.2 mm with a 3 μ particle size packing. Peak areas were automatically recorded as previously described (11).

Methyl esters were prepared by interesterification with methanol using the method of Christie (14). A Hewlett Packard Model 5840A gas chromatograph equipped with a 12′ x 0.125″ o.d. column of 10% Silar 10 C (Applied Science, State College, PA) on 100-200 mesh Gas Chrom Q was used. The column temperature was programmed from 200 to 225 C at 2 C/min with a helium flow rate of 10 ml/min.

Solvents

Methylene chloride was a reagent grade purchased by the University of Minnesota from local suppliers and was purified by a preliminary distillation followed by shaking it in a separatory funnel with concentrated sulfuric acid several times, then with dilute sodium carbonate and finally with water. The washed solvent was dried over calcium chloride and redistilled in an all-glass still.

Acetonitrile was a reagent grade obtained from Fisher Scientific Co., Fairlawn, NJ. It was fractionally distilled through a 2-meter Hyper-Cal Podbielniak column at a reflux ratio of 20:1, or mixed with phosphorous pentoxide and, after several days, distilled in an all-glass still at ca. 10 C under reduced pressure. Spectral-grade purity of acetonitrile was generally not required, but the above procedure was performed to remove any nonvolatile contaminants and to ensure a uniform solvent from batch to batch.

The chromatography was carried out with a linear gradient elution program starting with various concentrations, generally 20% by vol, of methylene chloride in acetonitrile, in which the concentration of methylene chloride was increased until all the components were eluted, or in an isocratic solvent system of different concentrations of methylene chloride in acetonitrile.

RESULTS

Chromatograms of the separation of reference mixture HPLC #G-1 of pure triglycerides by gradient and isocratic solvent systems are shown in Figures 1A and 1B, respectively. The analysis in Figure 1A was obtained with ODS Zorbax column I and elution with a linear gradient solvent system of acetonitrile and methylene chloride. The lowest chain length species of the saturates were eluted first with the highest concentration of acetonitrile. With the addition of more methylene chloride, the longer chain length saturated species were eluted. The unsaturated triglyceride species were eluted in reverse order depending on their degree of unsaturation, i.e. the more unsaturated species were eluted faster than those of lower unsaturation. Figure 1A also shows that the triglycerides of this mixture are separated with baseline efficiency using a gradient solvent system. The peaks are sharp and little band spreading occurs over the entire range of the mixture. The gradient and flow rate, as well as the column, were selected for their general applicability as well as for high resolution. Triglyceride species less polar than 54:0 can also be analyzed by allowing the gradient to go to a higher concentration of methylene chloride. This mixture also contains several

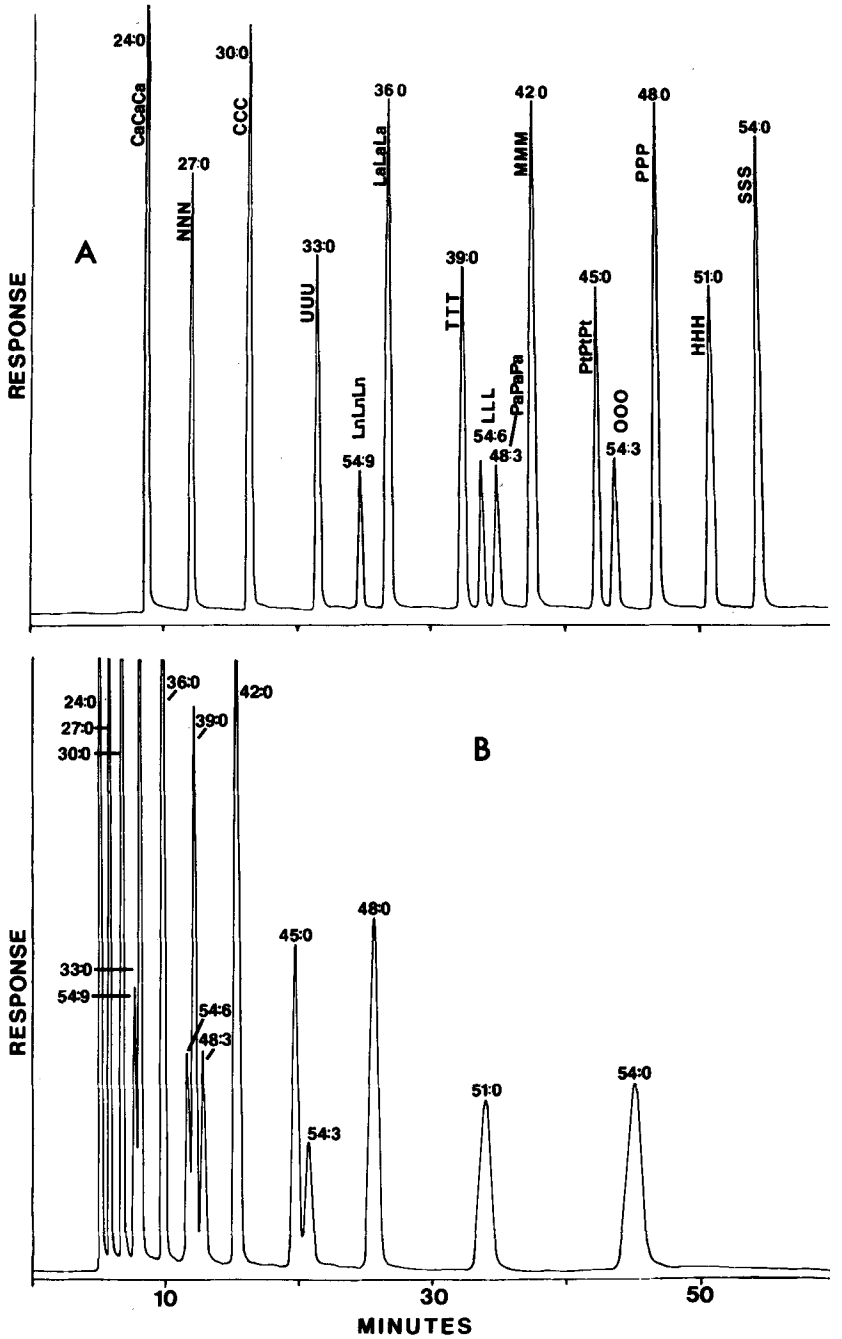

FIG. 1. Separation of triglyceride mixture HPLC #G-1; Column–250 X 4.6 mm, 5 μm, Zorbax C_{18} ODS with 15% carbon content (Column I); A) mobile phase–60 min linear gradient from 15 to 55% methylene chloride in acetonitrile; flow rate–0.8 ml per min; sample size–40 μg; detector–flame ionization. B) Isocratic elution with 45% methylene chloride in acetonitrile. Peaks: number before colon = number of carbon atoms in acyl chains, number after colon = number of double bonds in acyl chains. CaCaCa = tricaprylin, NNN = trinonanoin, CCC = tricaprin, UUU = triundecanoin, LnLnLn = trilinolenin, LaLaLa = trilaurin, TTT = tritridecanoin, LLL = trilinolein, PaPaPa = tripalmitolein, MMM = trimyristin, PtPtPt = tripentadecanoin, OOO = triolein, PPP = tripalmitin, HHH = triheptadecanoin, SSS = tristearin.

groups of critical pairs, 54:9/36:0; 54:6/48:3/42:0 and 54:3/48:0, making it a good overall mixture for testing the efficiency of ODS columns.

For comparison, Figure 1B shows the best separation of the components of mixture HPLC #G-1 that could be obtained isocratically with methylene chloride and acetonitrile. The initial peaks are sharp but loss of resolution, namely between 54:9-33:0, 54:6-39:0-48:3, and 45:0-54:3, and band spreading occur with the increase in retention time.

The log-linear plots of the retention time-equivalent carbon number (ECN) of the simple saturated triglycerides of the separation of the components of HPLC #G-1 by both the gradient (curve A) and isocratic (curve B) solvent systems are shown in Figure 2. The isocratic system generally gives a linear relationship (3-5) in a log-linear plot. Gradient solvent systems are like GC with temperature programming and cannot be expected to give a linear relationship in a log-linear plot. The curve shown in Figure 2 is highly reproducible and can be used just as well as a linear plot. Using this relationship, the constants for the calculation of TCN values for triglycerides containing 1, 2 and 3 double-bond acyl groups [oleate (0.68), linoleate (0.73) and linolenate (0.39)] were determined. The application of these constants to the determination of TCN values of the triglyceride species identified in olive oil, reference mixture F (Fig. 3) and other standards, is shown in Table 1. Although the TCN value is an empirical determination and is influenced by acyl group composition, the experimental and calculated values agree sufficiently

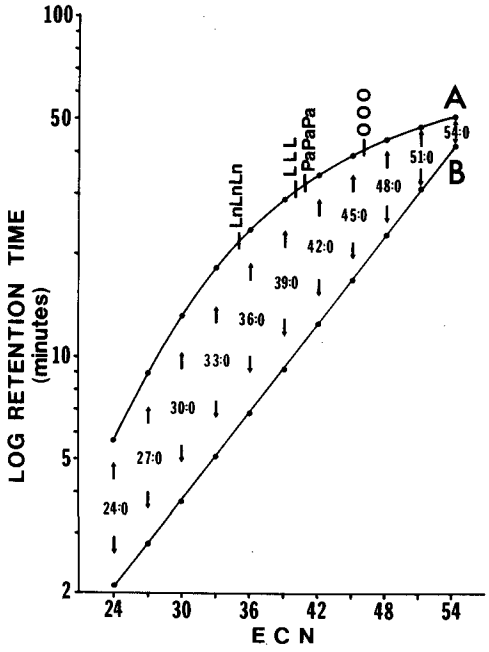

FIG. 2. Relationship of the log-linear plot of the ECN of the simple saturated triglycerides and retention time; Curve A = gradient elution, Curve B = isocratic elution. Identifications: number before colon = number of carbon atoms in acyl chains, number after colon = number of double bonds in acyl chains.

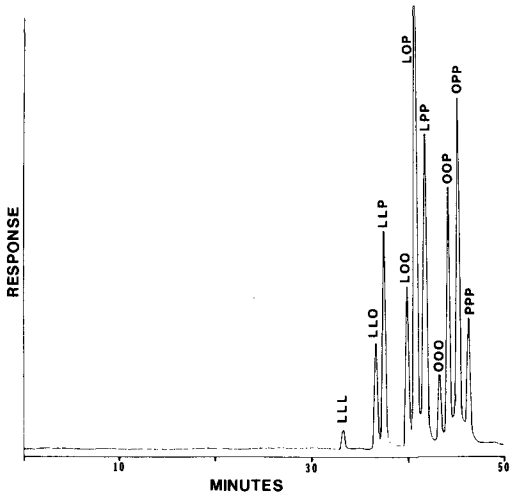

FIG. 3. Chromatogram of interesterification mixture F under the same conditions as in Figure 1A. P = palmitate, O = oleate and L = linoleate (the order of the designations does not indicate the separation of positional isomers).

TABLE 1

TCN Values of Triglyceride Species

Molecular species	Calculated*	Found
LLO	41.8	41.8
LnOO	42.2	42.1
LLP	42.5	42.3
LnOP	42.9	42.7
LOO	43.9	43.9
OOPa	44.2	44.3
LOP	44.6	44.5
OPaP	44.8	44.9
LPP	45.3	45.2
OOO	46.0	45.9
OOP	46.6	46.6
OPP	47.3	47.3
PPP	48.0	48.1
OOS	48.6	48.5
OPS	49.3	49.3
OSS	51.3	51.3

*TCN constants: Ln = 0.39, L = 0.73, O = 0.68, Pa = 0.45. Ln = linolenate, L = linoleate, O = oleate, Pa = palmitoleate, P = palmitate, S = stearate (the order of designation does not indicate positional isomers).

to be useful in the identification of triglyceride species.

In order to determine further the efficiency of the gradient system for the separation of triglyceride species, it was tested with the interesterification mixtures described in *Methods.* In reference mixture HPLC #G-1 (Fig. 1A), the species varied by differences of 3 carbon atoms or 3 double bonds. Figure 4 shows the separation of triglyceride species differing by 1 double bond (Fig. 4A) or 1 methylene group (Fig. 4B), mixtures B and E, respectively. The triglycerides differing by 1 double bond were separated better than those differing by 1 methylene group, but the components of both mixtures were separated in sharp peaks with little band spreading and, essentially, baseline efficiency.

The analysis of the 3 groups of 4 component critical pair mixtures, C, D and A, with ECN of 36, 42 and 48, respectively, is shown in Figure 5. These mixtures were added together for the analysis shown in Figure 5 and demonstrate the efficiency of the gradient system for the separation of 4 component critical pair mixtures.

Mixture G, whose 4 components differ by only one *trans* double bond, can be readily detected but are not completely separated as shown in Figure 6A. Figure 6B shows that separating some triglyceride species that differ in their acyl groups only by the position of the double bond (HPLC #G-3) is possible. These mixtures provide an indication of the limit of the efficiency of the column for the analysis of natural and partially hydrogenated fats.

Analysis of Olive Oil

The application of the gradient method to olive oil with column I and a combination of columns I and II is shown in Figure 7. The chromatogram obtained with column II (not shown) gave a better separation of the more polar species than column I, although the overall separation with column I shown in Figure 7 was better. When the 2 columns were connected together, the individual characteristics of each column were retained and the separation of both more polar and less polar components was superior, as shown in Figure 7B. The triglyceride species composition of olive oil is well known, hence, identification of the com-

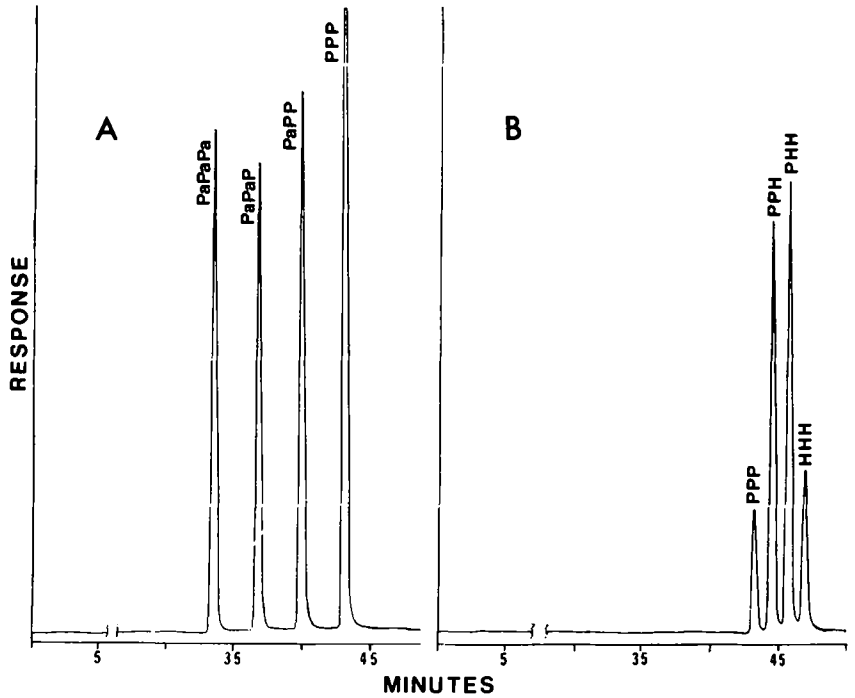

FIG. 4. Separation of interesterified test mixtures B and E under the same conditions as in Figure 1A. A = test mixture B, differences by one double bond; B = test mixture E, differences by one methylene group. (Pa = palmitoleate, P = palmitate and H = heptadecanoate; the order of the designations does not indicate the separation of positional isomers.)

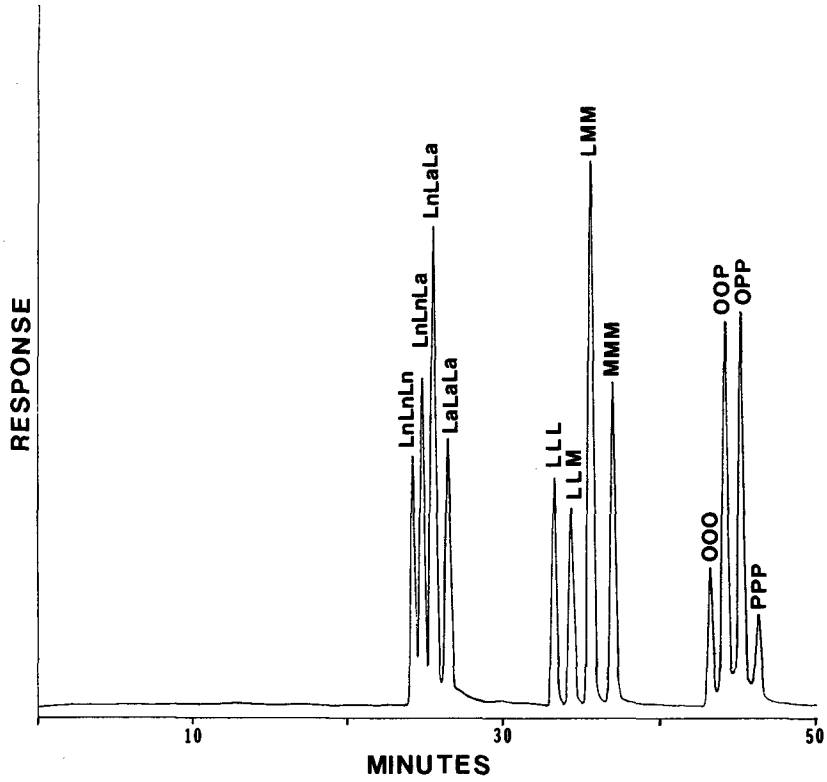

FIG. 5. Separation of a mixture of 3 groups of 4 component critical pairs with ECN's of 36, 42 and 48, triglyceride reference mixtures C, D and A, respectively. Conditions are the same as in Figure 1A. Ln = linolenate, La = laurate, L = linoleate, M = myristate, O = oleate and P = palmitate (the order of the designations does not indicate the separation of positional isomers).

ponents in Figure 7 was made by using references from the literature (4,5,7) that relate to the retention time of pure triglycerides, by comparing peak retention times to the components of mixture F, and by comparing calculated and experimental TCN values.

Figure 8 shows the analysis of olive oil with the third column using an isocratic solvent system at 2 different flow rates (1.2 and 0.6 ml/min). The best resolution was obtained with the slower flow rate, but the speed of the elution was increased proportionately by doubling the flow rate with this column. In either case, the minor components were not separated as well as with the longer columns. A gradient system was not worked out for this column, but might have increased both speed and resolution.

DISCUSSION

A major feature of the FID is that it permits the use of gradient elution, increasing the versatility of the chromatography. By using a gradient solvent system, triglyceride species can be separated on the basis of differences of one methylene group or one double bond. Two and 4 component critical pair mixtures were separated over the molecular weight range of the triglycerides of most common vegetable oils. Two columns connected together provide greater resolution than a single column but require approximately twice as long for an analysis, as shown in Figure 7. However, not only does the longer column provide greater resolution, it permits the use of larger loads, increasing the sensitivity for the detection and analysis of minor components, as shown in Figure 7. The long lead time and relatively short emergence time of the 2-column system demonstrated on olive oil should also facilitate the analyses of triglycerides containing highly polyunsaturated fatty acids that occur in some animal fats and fish oils.

The analysis of olive oil by both the single and double column systems gave results similar

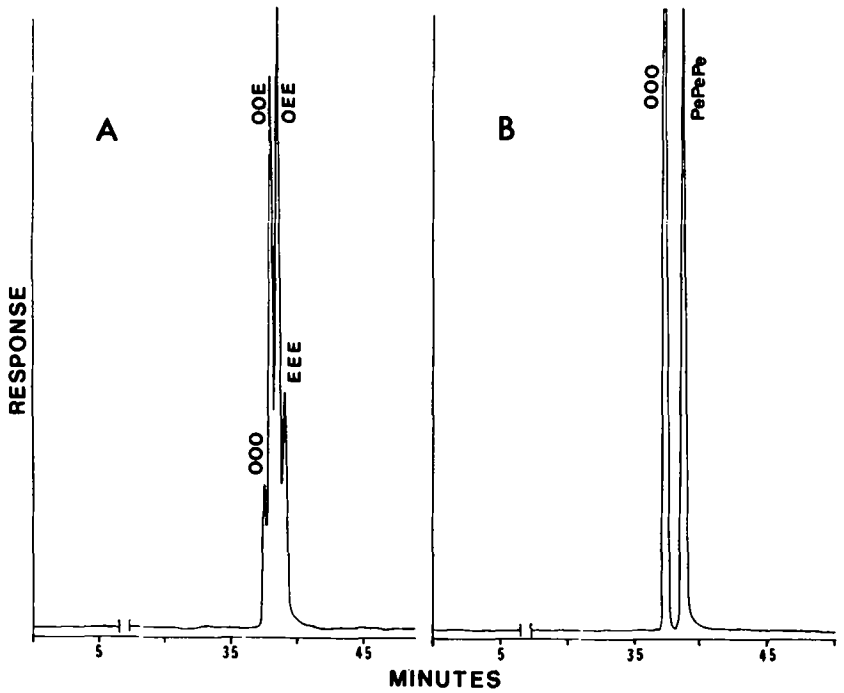

FIG. 6. Separation of interesterified test mixture G and triglyceride reference mixture HPLC #G-3, A and B, respectively. The conditions are the same as in Figure 1A. O = oleate, E = elaidate and Pe = petroselinate (the order of the designations in A does not indicate the separation of positional isomers).

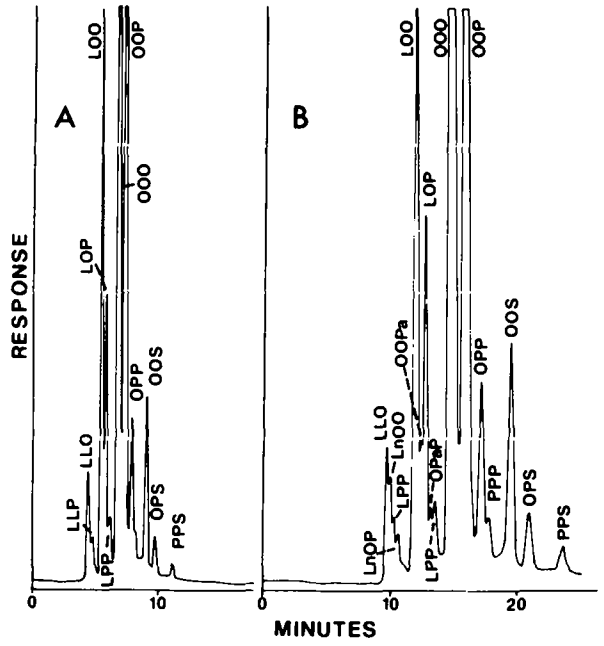

FIG. 7. Triglyceride species analyses of olive oil. A, the conditions are as in Figure 1A except that a gradient from 30 to 60% was used; B, Zorbax columns I and II were connected and a 120 min gradient used. Species identification as indicated where P = palmitate, S = stearate, O = oleate, L = linoleate, Ln = linolenate, Pa = palmitoleate and U = unidentified. The order of the designations does not indicate the separation of isomers.

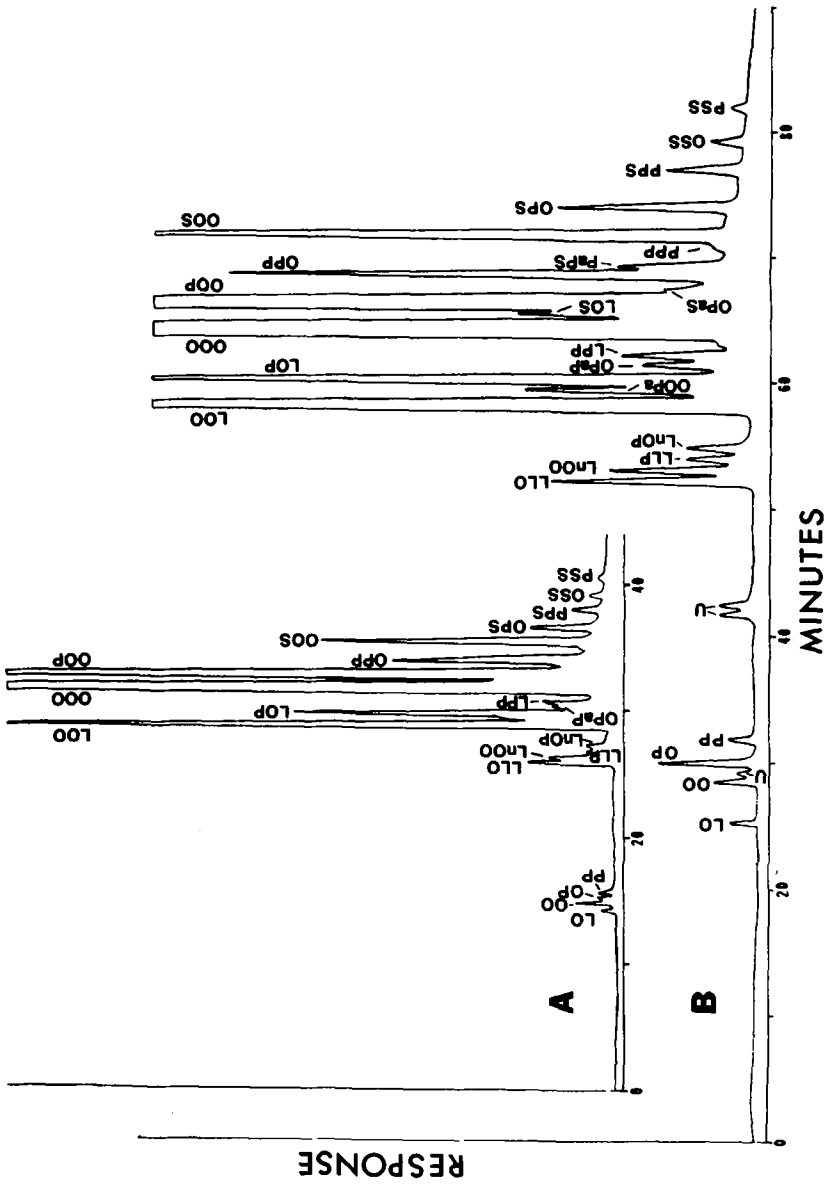

FIG. 8. The triglyceride species analysis of olive oil with Zorbax column III. Isocratic elution with 45% methylene chloride in acetonitrile; sample size 100 μg and FID with a 1:1 effluent split. A = flow rate of 1.2 ml/min and B = 0.6 ml/min.

to those reported by Dong and DiCesare (7) and El-Hamdy and Perkins (5) except in the analysis of the minor components, particularly linolenate species, which were readily detected. These components were well separated into sharp, distinct peaks by the 2-column systems, as shown in Figure 7. The resolution and identification of the linolenate species of olive oil is very important as the level of this fatty acid is used as a parameter for the detection of adulteration of this oil. Obtaining similar separations of these and other minor components with isocratic systems would be very difficult because fast elution times are virtually mandatory in these systems to avoid excessive peak broadening with a consequent loss in sensitivity, as well as resolution. Progressive peak broadening, which occurs with isocratic systems, has been demonstrated by Snyder et al. (15), on a theoretical basis as well as by Dong and DiCesare (7) with olive oil.

The most important factor in the analysis of natural oils is still the need for greater resolution. The FID provides advances for more efficient systems which are required for the complete physical separation of triglyceride species. Such an accomplishment does not seem to be outside of the realm of possibility in view of the versatility that can be obtained with gradient solvent systems and the continued improvement in ODS columns. Moreover, the triglyceride mixtures described here provide a good test for advances in this area. Although the identification of peaks by their TCN values is satisfactory for most oils, it is somewhat laborious without a computer and not always certain, especially in the case of minor components and those species not well separated. To obtain unequivocal identification by GLC of methyl esters of isolated fractions is laborious and not always possible because of the difficulty of collecting closely separated peaks. A more positive technique of identification is the combination of HPLC with chemical ionization/mass spectrometry as recently reported by Kuksis et al. (16) and as being developed in our laboratory (17). However, this technique is not quantitative, does not distinguish positional and geometric acyl groups and is generally more complex and expensive. Hence, the FID coupled with a high resolution column and a good library of reference compounds is the method of choice.

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REFERENCES

1. Nickell, E.C., and Privett, O.S. (1967) Sep. Sci. 2, 307-318.
2. Pei, P., Henley, R., and Ramachandran, S. (1975) Lipids 10, 152-156.
3. Plattner, R.D., Spencer, G.F., and Kleiman, R. (1977) J. Am. Oil Chem. Soc. 54, 511-515.
4. Plattner, R.D. (1981) Methods Enzymol. 72, 21-34.
5. El-Hamdy, A.H., and Perkins, E.G. (1981) J. Am. Oil Chem. Soc. 58, 867-872.
6. Perkins, E.G., Hendren, D.J., Pelick, N., and Bauer, J.E. (1982) Lipids 17, 460-463.
7. Dong, M.W., and DiCesare, J.L. (1983) J. Am. Oil Chem. Soc. 60, 788-791.
8. Stolyhwo, A., and Privett, O.S. (1973) J. Chromatogr. Sci. 11, 20-25.
9. Erdahl, W.L., Stolyhwo, A., and Privett, O.S. (1973) J. Am. Oil Chem. Soc. 50, 513-515.
10. Privett, O.S., Dougherty, K.A., Erdahl, W.L., and Stolyhwo, A. (1973) J. Am. Oil Chem. Soc. 50, 516-520.
11. Phillips, F.C., Erdahl, W.L., and Privett, O.S. (1982) Lipids 17, 992-97.
12. Phillips, F.C., and Privett, O.S. (1981) J. Am. Oil Chem. Soc. 58, 590-594.
13. Privett, O.S., and Erdahl, W.L. (1978) Anal. Biochem. 84, 449-461.
14. Christie, W.W. (1982) J. Lipid Res. 23, 1072-1075.
15. Snyder, L.R., Dolan, J.W., and Grant, J.R. (1979) J. Chromatogr., Chromatogr. Rev. 165, 3-30.
16. Kuksis, A., Marai, L., and Myher, J.J. (1983) J. Chromatogr. 273, 43-66.
17. Privett, O.S., and Erdahl, W.L. (1981) Methods Enzymol. 72, 56-108.

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COMMUNICATIONS

Alcohol Ingestion and Levels of Hepatic Fatty Acid Synthetase and Stearoyl-CoA Desaturase Activities in Rats

G. ANANDA RAO*, GAIL LEW and EDWARD C. LARKIN, *Hematology Research Laboratory, Veterans Administration Medical Center, 150 Muir Road, Martinez, CA 94553*

ABSTRACT

Male Sprague-Dawley rats were fed, ad libitum for 30 days, a fat-free (FF) liquid diet containing 34% of the calories as ethanol or a control FF diet in which alcohol was replaced by an isocaloric amount of dextrins. The cytosolic fatty acid synthetase and the microsomal stearoyl-CoA desaturase activities in the livers of rats fed the alcohol diet were about half of those observed in the livers of control rats. The conclusion is that chronic ethanol consumption depresses the activities of these lipogenic enzymes in the liver.
Lipids 19:151-153, 1984.

INTRODUCTION

Chronic ingestion of a diet in which 34% of the calories are ethanol and 35% are fat causes an accumulation of triacylglycerol (TG) in liver, resulting in fatty liver (1-3). Recent studies from this laboratory have shown that even when dietary fat is excluded from the diet, chronic ethanol consumption produces severe fatty liver (4). An increase in hepatic lipogenesis induced by alcohol may have resulted in fatty liver when animals were fed the fat-free (FF) diet (4). Dietary ethanol has been implicated as a cause in the increase in the synthesis of fatty acid in the liver (5,6). If ethanol consumption enhanced lipogenesis, whether it was from the increased availability of the substrates (acetate and reducing power) generated by its metabolism or was also affected by increased fatty acid synthetase (FAS) activity, was not known. To our knowledge, FAS levels have not been determined in the livers of animals given a chronic dose of ethanol.

Alcohol ingestion produces a decrease in the hepatic levels of Δ9 acyl-CoA desaturase (7,8). Since the assays were carried out in animals fed polyunsaturated fat diets, low levels of desaturase activity were observed (9). In the present study, we maintained rats on a FF diet, a condition known to enhance the tissue levels of both FAS and Δ9 acyl-CoA desaturase, and examined the effect of chronic ethanol consumption on these enzyme activities. A preliminary report on this study has already appeared (10).

MATERIALS AND METHODS

Animals and Diets

Male Sprague-Dawley rats weighing ca. 75 g were purchased from Hilltop Lab Animals, Inc., Chatsworth, CA. They were housed individually in stainless-steel cages. For 30 days, a group of 6 rats were fed ad libitum a Lieber/DeCarli liquid ethanol diet in which fat was replaced with an isocaloric amount of maltose-dextrins. Another group of 6 rats were fed for 30 days a Lieber/DeCarli control diet in which alcohol and fat were replaced by an isocaloric amount of maltose-dextrins. The diets were custom-made by Bio-Serv, Inc., Frenchtown, NJ. The overall composition of protein, fat, carbohydrate, fiber, vitamins and elements of Lieber/DeCarli liquid diets has been described recently (11). Rats in the control group were individually pair-fed with animals in the alcohol group.

Isolation of Cytosol and Microsomes from Liver

The rats were anesthetized by an intraperitoneal injection of sodium pentobarbital (170 mg/kg rat); the livers were quickly removed and rinsed with ice-cold 0.25 M sucrose. Pieces of liver (ca. 2 g) were homogenized in a Potter-Elvehjem tissue grinder with 3 volumes of ice-cold 0.25 M sucrose. All subsequent preparations were carried out at 0-4 C. The homogenate was centrifuged at 1,000 x g for 15 min. The resulting supernatant fraction was centrifuged at 100,000 x g for 1 hr. The cytosol (without the floating fat layer) was removed and its FAS activity measured. The microsomal pellet was washed, suspended in 0.154 M KCl and used for the assay of Δ9 acyl-CoA desaturase activity.

Enzyme Assays

The FAS activity was analyzed as described by Smith and Abraham by measuring the decrease in extinction at 340 nm that accompanies the oxidation of NADPH during the

*To whom correspondence should be addressed.

synthesis of fatty acids (12). Stearoyl-CoA desaturase activity was measured as described earlier (9,13). Protein concentrations of the cytosolic and microsomal fractions were determined by the method of Lowry et al. (14), using bovine serum albumin as a standard. Fatty acid synthetase activity was expressed as units/mg protein. A unit of FAS is the amount that catalyzes the malonyl-CoA dependent oxidation of 1 μ mole NADPH/min at 25 C. The stearoyl-CoA desaturase activity was given as nmol oleate produced/min/mg protein.

Materials

Lithium salts of acetyl-CoA and malonyl-CoA, NADPH, NADH, ATP, CoASH, GSH, and bovine serum albumin were purchased from Sigma Chemical Co., St. Louis, MO. Pure stearic acid was obtained from Applied Science, State College, PA. [1-^{14}C] Stearic acid was purchased from New England Nuclear Corp., Boston, MA, and purified by thin layer chromatography (>99%).

RESULTS AND DISCUSSION

Liver FAS Activity

In control rats, which were fed the FF diet for 30 days, the hepatic FAS activity was 41.43 units/mg protein (Table 1). This value is somewhat lower than that reported in the livers of mice, which were fed a FF diet for only 3 days (54.4 units/mg protein)(15). Although whether a species difference in the levels of liver FAS exists is not known, the lower values we observed could be caused by the type of regimen used in the present study. Control rats were pair-fed with those in the alcohol-diet group. Rats in the alcohol group, which were fed ad libitum, ate during the course of the day. On the other hand, those in the control group consumed their daily ration a few hours after feeding and fasted until they were fed the next day. We have observed that when rats were fed the control diets ad libitum, the consumption was ca. 2 times greater than when they were pair-fed with the alcohol group (3). The FAS activity in livers of control rats would probably have been higher if animals were fed the FF diet ad libitum.

In rats fed the FF diet containing ethanol, the level of hepatic FAS was ca. half of that in the controls (21.75 units/mg protein) (Table 1). In spite of this decrease, enzyme activity observed was several times greater than that found in rats fed an alcohol diet containing fat. For example, in parallel experiments, when 6 male Sprague-Dawley rats were fed for 30 days a Lieber/DeCarli liquid ethanol diet containing 35% calories as fat, the liver FAS activity was found to be low (5.1 ± 0.8 units/mg protein). In those fed the Lieber/DeCarli control diet, liver FAS activity was greater than in those fed the alcohol diet (7.75 ± 1.11 units/mg protein). Therefore, even when fat was present in the diet, chronic ethanol consumption produced a significant ($P < 0.01$) reduction in liver FAS activity. The levels of FAS in the livers of rats fed the FF diets suggest that, although the hepatic lipogenic rate may be reduced by alcohol ingestion, it could be several times greater than in the livers of rats maintained on a high-fat diet.

Most of the fatty acid of liver TG in animals given a chronic dose of ethanol are of dietary origin (3,16) and the 2-monoglyceride backbone of diet fat is, for the most part, retained during the development of fatty liver (17). Thus, the fatty liver observed in animals is mainly caused by the accumulation of dietary TG. However, the TG in the fatty livers found in rats fed a FF diet containing ethanol must originate from lipogenesis (4). As indicated by the levels of FAS, hepatic lipogenesis in animals fed a FF diet with ethanol could be several times greater than in those fed diets containing fat. The increase in lipogenesis may be related to the absence of fat rather than the presence

TABLE 1

Liver Cytosolic Fatty Acid Synthetase and Microsomal Stearoyl-CoA Desaturase Activities of Rats Fed a Fat-free Diet with or without Ethanol

Diet	Fatty acid synthetase units/mg protein	Stearoyl-CoA desaturase nmole oleate produced/min/mg protein
FF – alcohol	41.23 ± 2.04	2.42 ± 0.12
FF + alcohol	21.75 ± 1.08	1.31 ± 0.03

Values given are as mean ± SE. These were obtained by duplicate determinations with each enzyme preparation from 6 rats in each group. In the case of both enzyme activities, the value obtained with the FF + alcohol group is significantly ($p < 0.001$) different compared with the corresponding value in the FF – alcohol group.

of alcohol in the diet. However, increased lipogenesis may not be the main cause for the production of fatty liver since, in control rats, although the hepatic FAS activity is 2-times greater (Table 1), only a small level of TG is present in the livers (4). In animals fed a FF ethanol diet, liver TG must arise from enhanced lipogenesis (when compared with fat-fed animals). However, whether fat is ingested or not, alcohol-induced fatty liver must be caused by an inhibition of fat mobilization from the liver.

Hepatic Stearoyl-CoA Desaturase Activity

The analysis of the relative levels of monoenoic fatty acids to saturated acids of liver lipids had suggested that hepatic Δ9 desaturase levels may be reduced by chronic ethanol ingestion (3). Reitz and coworkers have found that chronic ethanol feeding does produce a reduction in hepatic microsomal stearoyl-CoA desaturase activity (7,8). While the enzyme activity in control rats was 0.56 ± 0.06 units, it was significantly reduced in alcohol-fed animals (0.16 ± 0.07 units) (8). Since the animals were fed a FF diet in the present study, the desaturase activity in control rats was several times greater than in those observed by Reitz and coworkers (2.42 ± 0.12 units) (Table 1). However, this level of activity was only about half of that reported in rats fed a FF diet ad libitum (9,13). As discussed in the case of FAS, this may be related to the type of dietary regimen used in the present study. Ingestion of alcohol caused a significant ($P < 0.001$) reduction in the desaturase activity (1.31 ± 0.03 units) in the livers of rats fed the FF diet. These observations show that chronic ethanol consumption reduces liver Δ9 desaturase activity.

In some neoplastic tissues and in physiological conditions such as starvation, when feeding a diet containing either no fat or high levels of polyunsaturated fat, the activities of FAS and Δ9 acyl-CoA desaturase either increase or decrease together (18-20). The results from the present study demonstrate that the adaptive changes in the 2 lipogenic enzymes are also comparable in the case of chronic ethanol consumption.

Earlier studies have shown that several hepatic enzymes related to lipogenesis (pyruvate dehydrogenase, ATP-Citrate lyase, acetyl-CoA synthetase, malic enzyme and glucose 6-phosphate dehydrogenase) are significantly reduced by prolonged alcohol consumption (21). Results from the present study demonstrate that the lipogenic enzymes, FAS and Δ9 acyl-CoA desaturase are also markedly reduced in the livers of rats given a chronic dose of alcohol. Further studies should reveal whether the reduction of the enzyme activities is caused by a decrease in the amount of enzyme protein or an inhibitory effect by ethanol or its metabolic products.

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REFERENCES

1. DeCarli, L.M., and Lieber, C.S. (1967) J. Nutr. 91, 331-336.
2. Stanko, R.T., Mendelow, H., Shinozuka, H., and Adibi, S.A. (1978) J. Lab. Clin, Med. 92, 228-235.
3. Goheen, S.C., Larkin, E.C., Manix, M., and Rao, G.A. (1980) Lipids 15, 328-336.
4. Goheen, S.C., Larkin, E.C., and Rao, G.A. (1983) Lipids 18, 285-290.
5. Lieber, C.S. (1974) Lipids 9, 103-116.
6. Reitz, R.C. (1979) Prog. Lipid Res. 18, 87-115.
7. Wang, L., and Reitz, R.C. (1980) Fed. Proc. 39, 541.
8. Reitz, R.C., Wang, L., Schilling, R.J., Starich, G.H., Bergstrom, J.D., and Thompson, J.A. (1981) Prog. Lipid Res. 20, 209-213.
9. Rao, G.A., and Abraham, S. (1975) Lipids 10, 835-839.
10. Rao, G.A., Lew, G., Larkin, E.C., and Goheen, S.C. (1983) Fed. Proc. 42, 1976.
11. Lieber, C.S., and DeCarli, L.M. (1982) Alcoholism: Clin. Exp. Res. 6, 523-531.
12. Smith, S., and Abraham, S. (1975) Methods Enzymol. 35, 65-74.
13. Rao, G.A., Crane, R.T., and Larkin, E.C. (1983) Lipids 18, 573-575.
14. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
15. Schwartz, R.S., and Abraham, S. (1982) Biochim. Biophys. Acta 711, 316-326.
16. Lieber, C.S., Spritz, N., and DeCarli, L.M. (1966) J. Clin. Invest. 45, 51-62.
17. Rao, G.A., Lew, G., and Larkin, E.C. (1983) Subst. Alcohol Action Misuse 4, 355-359.
18. Lee, T-C., Stephens, N., and Snyder, F. (1974) Cancer Res. 34, 3270-3273.
19. DeTomas, M.E., Mercuri, O., and Peluffo, R. (1975) Lipids 10, 360-362.
20. Abraham, S., and Rao, G.A. (1976) in Control Mechanisms in Cancer (Criss, W.E., Ono, T., and Sabine, J.R., eds.) pp. 363-378, Raven Press, New York.
21. Salvolainen, M.J., Hiltunen, J.K., and Hassinen, I.E. (1977) Biochem. J. 164, 169-177.

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Effect of Different Amino Acid Diets on Δ 5, Δ 6 and Δ 9 Desaturases

RAUL O. PELUFFO[1,*], ANIBAL M. NERVI[1], MARIA S. GONZALEZ and RODOLFO R. BRENNER[1], *Instituto de Investigaciones Bioquimicas de La Plata (INIBIOLP), UNLP-CONICET, Facultad de Ciencias Medicas, calles 60 y 120, 1900-La Plata, Argentina*

ABSTRACT

A high-protein diet with 45% calories from casein increased Δ6 desaturase activity in rat-liver microsomes. High-protein diets with 45% calories from a synthetic mixture of amino acids in the same proportion as casein decreased the Δ9 desaturase, slightly increased the Δ5 desaturase and greatly increased Δ6 desaturase activities compared with a high-carbohydrate diet. The elimination of phenylalanine and tyrosine from the synthetic mixtures of amino acids increased the Δ6 desaturase activity. Massive amounts of phenylalanine or tyrosine in the diet inhibited Δ6 desaturase activity. Tyrosine and phenylalanine may, by conversion to tyrosine, decrease the activity of the Δ6 desaturase.
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INTRODUCTION

The biosynthesis of polyunsaturated acids, especially the activity of the desaturases, is modified by the composition of the diet. The activity of the Δ6 desaturase of rat-liver microsomes depends on the proportion of proteins in the diet. High-protein diets have been shown to increase the activity of the enzyme (1-2). A diet in which 40% of the calories are from casein increases the Δ6 desaturase activity ca. 30% compared with a normally balanced diet containing less than 25% casein. However, high-protein diets do not modify the Δ9 desaturase activity of rat liver (3). The effect of such diets on the activity of Δ5 desaturase has not been carefully studied. However, a casein hydrolysate has been shown to decrease the activity of the Δ5 desaturase in minimal deviation hepatoma (HTC) cells that were incubated in a medium without carbon (4).

The basic mechanism for activating the Δ6 desaturase is not yet understood. Whether the activation is because of a general effect of the proteins or is stimulated by particular amino acids is also unknown. To study this problem, different groups of rats were fed with isocaloric synthetic diets from which specific amino acids had been excluded. Phenylalanine and tyrosine were shown to inhibit the Δ6 desaturase whereas the Δ9 and Δ5 desaturases were apparently not modified.

MATERIALS AND METHODS

Two experiments were performed. In the first experiment, female Wistar rats, each weighing 150 g, were separated into several groups of 5 animals. The rats fasted for 24 hr and were then fed the following diets for 24 hr (expressed in the percentage of calories): Group 1-10% sunflower oil, 85% sucrose and 5% casein; Group 2-10% sunflower oil, 45% sucrose and 45% casein; Group 3-10% sunflower oil, 45% sucrose and 45% synthetic mixure of amino acids (grams of L-amino acids per 100 g of mixture: Gly.=2.01; Ala.=3.48; Ser.=6.4; Thr.=4.2; Pro.=8.24; Val.=5.70; Ile.=5.75; Leu.=7.90; Phe.=4.66; Tyr.=7.30; Trp.=2.0; Cy/2=0.44; Met.=2.67; Asp.=8.05; Glu.=21.0; Gln.=1.65; Arg.=3.92; His.=2.92; Lys.=9.31); groups 4-9 the same diet as group 3 except that 2 or 3 essential amino acids were excluded and the same protein ratio (45%) was maintained by increasing proportionally the other amino acids (Table 1). Experiments using groups 3-9 were carried out at different times, and groups 1 and 2 were always the controls.

In the second experiment, 5 groups of 5 female rats were used. They fasted for 24 hr and were fed for another 24 hr with the following diets (expressed in percent calories): Group 1-10% sunflower oil, 5% casein and 85% sucrose; group 2-10% sunflower oil, 25% casein and 65% sucrose. In groups 3, 4 and 5, 20% casein was replaced by phenylalanine, tyrosine and tryptophan, respectively. No vitamin or mineral mixtures were added to the diets.

At specified times the rats were killed by decapitation and the livers were excised. The liver microsomes were isolated as described previously (5).

In the first experiment, Δ9, Δ6 and Δ5 desaturases were determined by incubating 100 nmol of [1-^{14}C] palmitic acid, 80 nmol of [1-^{14}C] linoleic acid and 100 nmol of [1-^{14}C] eicosa-8,11,14-trienoic acid, respectively, with 5 mg of microsomal proteins and the necessary cofactors (6) at 35 C for 15 min in a final volume of 1.6 ml. New England Nuclear, Boston, MA, provided [1-^{14}C] palmitic acid (56 mCi/mmol, [1-^{14}C] linoleic acid (55 mCi/mmol) and [1-^{14}C] eicosa-8,11,14-trienoic acid (61 mCi/

[1]Members of the Carrera del Investigador Científico, Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina.
*To whom correspondence should be addressed.

TABLE 1

Effect on Δ9, Δ6 and Δ5 Desaturases by Elimination of Essential Amino Acids from the Diet

Diet Composition (calorie)	Groups								
	1	2	3	4	5	6	7	8	9
Sunflower oil	10	10	10	10	10	10	10	10	10
Sucrose	85	45	45	45	45	45	45	45	45
Casein	5	45		–					–
Amino acid mixture		-	45	45	45	45	45	45	45
Excluded amino acids[a]				Met Trp Lys	Val Phe Tyr	Leu Ile	Arg His Thr	Phe Tyr	Phe Tyr
Fatty acid desaturation									
% Desaturation of [1-^{14}C] palmitic acid	30.8 ± 3.2 P<0.001	15.0 + 1.4	14.1 ± 1.4	12.1 ± 1.5	12.9 ± 1.0	15.3 ± 1.6	16.7 ± 2.1	12.1 ± 1.3	14.4 ± 1.1
% Desaturation of [1-^{14}C] linoleic acid	10.0 ± 1.4 P<0.001	26.5 ± 1.6	25.9 ± 1.3	25.4 ± 1.5	40.4 ± 2.2 P<0.001	26.2 ± 1.7	25.3 ± 1.6	42.6 ± 2.4 P<0.001	41.8 ± 2.1 P<0.001
% Desaturation of [1-^{14}C] eicosa-8,11, 14-trienoic acid	10.2 ± 1.0 P<0.01	16.4 ± 1.5	16.5 ± 1.6	16.6 ± 1.9	17.3 ± 1.8	17.8 ± 1.9	16.4 ± 1.3	17.6 ± 1.7	17.4 ± 1.8

Results are the mean of 5 animals ± S.E.

P=values refer to group 2.

[a]Met=Methionine; Trp=Tryptophan; Lys=Lysine; Val=Valine; Phe=Phenylalanine; Tyr=Tyrosine; Leu=Leucine; Ile=Isoleucine; Arg=Arginine; His=Histidine; Thr=Threonine.

mmol). After incubation, the fatty acids were saponified and esterified. The percentage of desaturation was measured by gas-liquid radiochromatography in a Packard apparatus with a proportional counter (6). In the second experiment, following the same procedure, only the activity of the Δ6 desaturase was determined.

RESULTS AND DISCUSSION

Table 1 shows the caloric composition of the diet administered to each group and the Δ9, Δ6 and Δ5 desaturase activities of the animals fed with the corresponding diet. Groups 2 and 3, which received 45% calories as casein or a synthetic amino-acid mixture, respectively, had the same Δ9, Δ6 and Δ5 desaturase activity. The result shows that the effect of casein was caused by the amino-acid composition.

The Δ9 desaturase showed increased activity only in group 1. This increase was probably caused by the high sucrose content of the diet (85% of calories) since high carbohydrate diets have been shown to activate the Δ9 desaturase (1-7), whereas high-protein diets do not modify Δ9 desaturase activity when compared with commercial diets containing 25% protein (3). Compared with the other groups, Δ5 desaturase activity decreased slightly in group 1. Since the diet of group 1 was a high-carbohydrate diet and, according to Jeffcoat and James (8), carbohydrates do not affect Δ5 desaturase, activation of the enzyme may be presumed to be caused by the high-protein composition of the diet. Besides, the elimination of different essential amino acids (groups 4 to 9) from the synthetic mixture of amino acids did not modify either Δ9 or Δ5 desaturase activity.

Table 1 shows that the Δ6 desaturase activity decreased remarkably in group 1 when compared with other groups fed a high-protein diet. This result was apparently produced by a double effect: inhibition in group 1 caused by the high-carbohydrate diet and the activation induced in the other groups by the high-protein diets.

The Δ6 desaturase activity increased greatly in the amino-acid synthetic diet where phenylalanine and tyrosine were eliminated (groups 5, 8 and 9) compared with group 3, which contained all the amino acids, and groups 4, 6 and 7, where other essential amino acids were eliminated.

These results suggest that phenylalanine and tyrosine inhibit the Δ6 desaturase. Consequently, a second experiment was carried out where massive doses of phenylalanine and tyrosine were tested (Table 2). The caloric distribution of the diet administered to group 1 was similar to a normal diet. Group 2 was the same as group 1 in experiment 1. In groups 3, 4 and 5, 20% of the calories supplied by casein in group 1 were replaced by phenylalanine, tyrosine and tryptophan, respectively. Tryptophan was chosen in group 5 since, like phenylalanine and tyrosine, it is a gluco- and ketogenic amino acid. In spite of the elimination of tryptophan in experiment 1 (group 4), the Δ6 desaturase activity did not change.

Table 2 shows a decrease in the Δ6 desaturation of linoleic acid in group 2 compared with group 1, an effect apparently produced by an inhibition of the enzyme by the high-carbohydrate diet of group 2. When 20% of the calories from casein were replaced by phenylalanine or tyrosine (groups 3 and 4), Δ6 desaturase activity was inhibited ca. 35% and 70%, respectively. No inhibition was observed when 20% casein was replaced by tryptophan.

Comparing results in Tables 1 and 2 suggests that tyrosine per se or a tyrosine metabolite inhibits Δ6 desaturase. Since the effect caused by phenylalanine is less than the effect of tyrosine, and phenylalanine is a precursor of tyrosine, the effect of phenylalanine may be produced after the conver-

TABLE 2

Effects of Massive Doses of Phenylalanine and Tyrosine on Δ6 Desaturase

% Composition of diet (calorie)	Groups				
	1	2	3	4	5
Sunflower oil	10	10	10	10	10
Casein	25	5	5	5	5
Sucrose	65	85	65	65	65
Phenylalanine	—	—	20	—	—
Tyrosine	—	—	—	20	—
Tryptophan	—	—	—	—	20
% Desaturation of [1-^{14}C] linoleic acid	17.8 ± 1.5	6.1 ± 0.3 P<0.001	11.9 ± 0.6 P<0.01	5.8 ± 0.4 P<0.001	18.2 ± 1.4

Results are the mean of five animals ± S.E.
P=values refer to group 1.

sion to tyrosine.

Although the mechanism of the effect of these amino acids on the Δ6 desaturation has not been investigated yet, phenylalanine and tyrosine convert to adrenaline, and adrenaline activates the formation of 3,5-cyclic AMP, which is also an inhibitor of Δ6 desaturase (9,10).

The results in Tables 1 and 2 suggest that the effect of high-protein diets on Δ6 desaturase, if the effect is of metabolic origin, is caused by metabolites produced by many amino acids.

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REFERENCES

1. Inkpen, C.A., Harris, R.R., and Quackenbush, F. (1969) J. Lipid Res. 10, 277-282.
2. Peluffo, R.O., Gómez Dumm, I.N.T.de, Alaniz, M.J.T.de, and Brenner, R.R. (1971) J. Nutr. 101, 1075-1083.
3. Peluffo, R.O., and Brenner, R.R. (1974) J. Nutr. 104, 894-900.
4. Alaniz, M.J.T.de, Gómez Dumm, I.N.T.de, and Brenner, R.R. (1982) Mol. Cell. Biochem. 48, 15-18.
5. Brenner, R.R., and Peluffo, R.O. (1966) J. Biol. Chem. 214, 5213-5219.
6. Nervi, A.M., Peluffo, R.O., Brenner, R.R., and Leikin, A.I. (1981) Acta Physiol. Latinoam. 31, 119-128.
7. Mercuri, O.F., Peluffo, R.O., and De Tomás, M.E. (1974) Biochim. Biophys. Acta 369, 264-268.
8. Jeffcoat, R., and James, A.T. (1977) Lipids 12, 469-474.
9. Gómez Dumm, I.N.T.de, Alaniz, M.J.T.de, and Brenner, R.R. (1975) J. Lipid Res. 16, 264-268.
10. Alaniz, M.J.T. de, Gómez Dumm, I.N.T.de, and Brenner, R.R. (1975) Mol. Cell. Biochem. 12, 3-8.

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Effect of Dietary Cholesterol Protected Against Ruminal Hydrogenation on the Plasma Cholesterol and Liver of Sheep

J.R. ASHES[*,a] **L.J. COOK**[1] **and G.S. SIDHU**[b], [a]*Commonwealth Scientific and Industrial Research Organization, Division of Animal Production, PO Box 239, Blacktown, NSW, 2148, and* [b]*Commonwealth Scientific and Industrial Research Organization, Division of Food Research, PO Box 52, North Ryde, NSW, 2113*

ABSTRACT

Dietary supplements containing cholesterol or sunflower oil were prepared to protect them against degradation in the rumen. On feeding daily supplements containing 1-2 g protected cholesterol and/or 100 g protected sunflower oil to sheep, along with a basal ration of crushed oat grain and lucerne chaff, a rise in the plasma cholesterol was observed when compared with control animals. Livers from sheep fed protected cholesterol were enlarged, friable and cirrhotic in appearance and contained large deposits of esterified and free cholesterol, while livers from animals fed protected sunflower oil alone contained much less cholesterol. Octadecenoates constituted the major fatty acids in cholesteryl esters, which, in animals fed protected sunflower oil, were mainly polyunsaturated. The factors involved in the deposition of liver lipid at very low dietary cholesterol concentrations (0.11-.22%) in sheep compared with monogastric animals are discussed. *Lipids* 19:159-163, 1984.

INTRODUCTION

Conventional diets of ruminants contain very low amounts of fat and cholesterol (1,2). However, when included in ruminant diets, cholesterol is readily hydrogenated in the rumen through microbial action (3,4). Consequently, the amount of cholesterol reaching the small intestine for absorption from dietary sources is determined by rumen microbial activity and the amount of cholesterol originally present in the diet (4,5).

Certain differences have been reported in the metabolism of dietary and endogenously synthesized cholesterol between ruminants and monogastric animals. While the liver contributes greatly to the total cholesterolgenesis in the monkey (6), rat (7), chicken (8) and man (9), it plays a relatively unimportant role in this respect in ruminants such as goats (10) and sheep (11). Diets relatively rich in fat (over 7%) cause an elevation in the concentration of blood plasma cholesterol in ruminants (2). This elevation seems to result from an increased demand for cholesterol to transport fat from the intestine to other tissues; to meet this demand, cholesterol synthesis in the epithelial cells of the small intestine is stimulated (12). On the other hand, incorporating polyunsaturated lipids in the diets of man (13,14) and rabbits (15) lowers the concentration of circulating cholesterol in the blood plasma.

Hypercholesterolemia and the deposit of cholesteryl esters have been observed in livers and other organs when relatively high concentrations of cholesterol, ranging from 0.5 to 1%, are included in the diets of monogastric animals (16,17), nonruminating calves (18) and ruminants (10,19-21). Since substantial amounts of dietary cholesterol in ruminants are hydrogenated and rendered unavailable for absorption, the effects produced by feeding the same levels of cholesterol to monogastric animals and ruminants could be misleading.

Cholesterol has been shown (3) to be protected against ruminal hydrogenation by encapsulating emulsified oil droplets containing cholesterol in a matrix of formaldehyde-treated protein. When fed in this form, cholesterol is readily absorbed in lactating goats and cows and suppresses the transfer of long-chain fatty acids from blood plasma to milk fat (22). This suppression occurs at the dietary concentrations of 0.015 to 0.1% cholesterol. This result indicates that feeding cholesterol in a protected form at a relatively low dietary concentration is capable of causing gross alterations in lipid metabolism in goats and cows. The present study was undertaken to study the effects of feeding protected (PrC) and unprotected (UPrC) cholesterol on the liver and plasma lipid metabolism of sheep fed lipid-rich or conventional low-lipid diets.

MATERIALS AND METHODS

Feeding and Management of Animals

For the first experiment, 8 one-year-old sheep (Merino X Border Leicester) were divided randomly into 3 groups of 3, 3 and 2 animals. The sheep were housed in individual pens and each was fed a basal ration of 800 g pellets containing alfalfa hay and crushed oats (1:1) daily for a preexperimental period of 5 weeks. This preexperimental period was followed by a feeding period of 10 weeks, each sheep in the first group receiving, in addition to the

[1]Deceased.

*To whom correspondence should be addressed.

basal ration, 8 g protected cholesterol supplement, containing the equivalent of 1 g PrC, mixed with 100 g crushed oats daily. The second group of sheep received an equivalent amount of UPrC fed in a manner similar to the first group. The third group of 2 sheep was used as a control and each animal received 100 g crushed oats daily in addition to the basal ration.

The first feeding period was followed by another period of 10 weeks when the quantity of PrC and UPrC was increased to the equivalent of 2 g cholesterol daily. All animals had free access to drinking water.

In a second experiment lasting 12 weeks, 2 groups of 3 sheep were used. Each animal in the first group received 250 g/day of protected sunflower supplement containing the equivalent of 100 g protected sunflower oil (PrO), 2 g/day PrC and the basal ration containing 200 g crushed oats and 400 g alfalfa chaff. The second group received PrO and the basal ration but no cholesterol.

PrC and UPrC Supplements

Methods described by Ashes et al. (3) were used for preparing these supplements. To produce PrC, cholesterol (Labchem grade, Ajax Chemicals, Sydney, Australia) was dissolved in chloroform (2:1, w/w) and mixed with sunflower oil containing 0.02% butylated hydroxyanisole. This mixture was emulsified with a 16% aqueous solution of casein at pH 8.5 (ratio casein/oil/cholesterol, 4:3:1, w/w/w). The emulsion was treated with formaldehyde (3.6 g/100 g casein) and the resultant gel was dried in a fluid bed drier at 50 C until all traces of chloroform were removed. UPrC was produced by mixing cholesterol suspended in sunflower oil with sodium caseinate in the same ratio as PrC.

PrO Supplement

The method of Gulati et al. (22) was used to prepare this supplement, containing 40% lipid. Of the total fatty acids in this lipid, 65% were polyunsaturated, 18.5% monounsaturated and the remainder saturated.

Sampling Procedures

Plasma. Blood samples, obtained from each animal at regular intervals from the jugular vein, were collected in heparinized tubes and centrifuged at 2,000 g for 15 min. The plasma was stored at −20 C until required for analysis.

Liver. After completion of the feeding experiment, the animals were slaughtered; their livers were removed, wiped clean of blood, weighed and examined for gross morphological changes. Representative samples of liver tissue were frozen at −20 C until used for analysis.

Thin-layer chromatography (TLC) of lipids. Liver lipids were extracted using the method of Bligh and Dyer (23) and examined by TLC adsorbosil 1 plates. The plates (0.5 mm thickness) were developed in a petroleum spirit (40-60 C BP)/diethyl ether/acetic acid system (84:15:1) and visualized after spraying with an ethanolic solution of 2,7-dichlorofluoroscein under ultraviolet light. For quantitation, the plates were streaked with up to 50 mg of lipid and developed and visualized as above. The bands were identified by Rf comparison with lipid standards (Applied Science, State College, PA), removed and extracted with 3 x 10 ml of diethyl ether, except the phospholipid band, which was extracted with 3 x 10 ml of chloroform/ethanol/water/acetic acid (65:50:10:1) and lipid classes determined gravimetrically.

The mean recovery of total lipid from TLC plates was 93.1 ± 0.5%. The ratio of cholesteryl ester to free cholesterol was calculated.

Cholesterol analysis. Total cholesterol in the plasma and liver extracts was determined by a Technicon Method (24).

Fatty acid analysis. Methyl esters of fatty acids were prepared using the method of Scott et al. (25) and analyzed and identified by gas-liquid chromatography according to the procedures outlined by Ashes et al. (26).

Statistical analysis. Significant differences between the means of the experimental treatments were determined by either of 2 methods. In the first experiment, analysis of variance was applied using the criterion of least significant difference for comparisons between the means (27). In the second experiment, Student's *t*-test was applied (27). When comparing plasma cholesterols, the mean for each period was obtained from the last 4 weeks of each treatment.

RESULTS AND DISCUSSION

Changes in the plasma cholesterol concentrations in the 3 groups of sheep in the first experiment are shown in Figure 1. The sheep fed 1 g PrC daily showed a significant ($P<0.05$) rise in plasma cholesterol concentrations after the first 10 weeks of feeding. The group fed 1 g UPrC daily maintained plasma concentrations that were not significantly different from the control group. However, when dietary levels of UPrC were raised to 2 g daily, plasma cholesterol concentrations began to rise and were significantly different ($P<0.05$) from the control animals at the end of the 10-week period. This result indicates that at this dietary concentration cholesterol was not being completely hydrogenated in the rumen and that amounts equivalent to feeding 1 g PrC/day were reaching the small intestine for absorption. On increasing dietary PrC to 2 g daily, the plasma concentrations continued

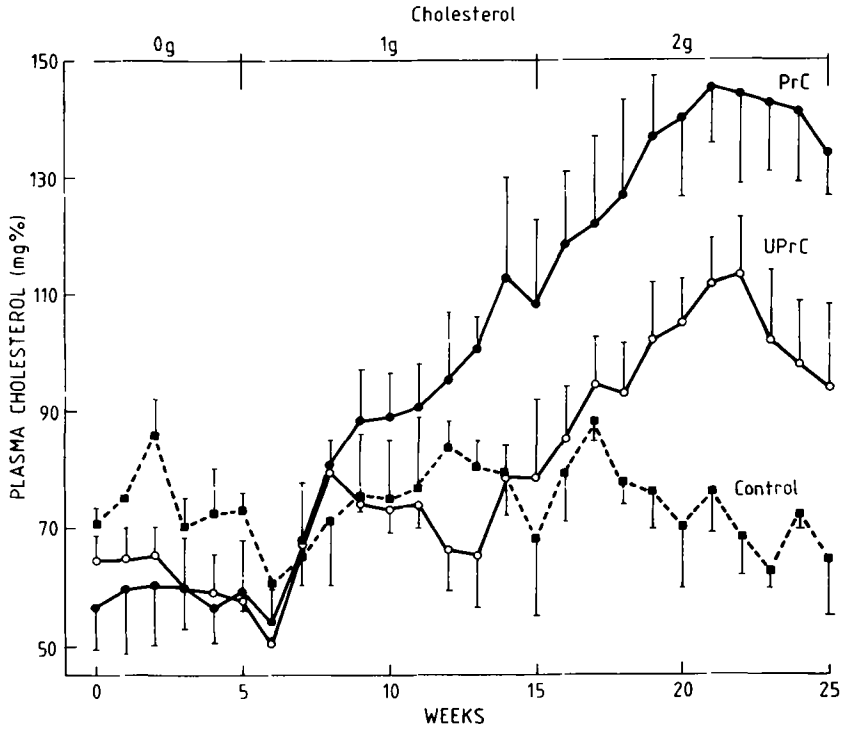

FIG. 1. Plasma cholesterol concentrations (±standard errors) with protected cholesterol (PrC) or unprotected cholesterol (UPrC) at dietary levels of 0, 1 and 2 g cholesterol per day.

to rise. Toward the end of the second 10-week feeding period, plasma cholesterol concentrations in both groups reached a plateau and tended to decrease.

The livers from the sheep fed PrC in the first experiment were greatly enlarged, very friable and cirrhotic in appearance. The bile ducts were enlarged and choked, indicating gross impairment of liver function in contrast to the livers of sheep from the control group, which appeared to be normal. A slight impairment of livers of sheep in the UPrC-fed group was apparent as indicated by a paler color relative to those in the control group. Table I shows the weights of livers and their contents of total lipids and cholesterol and change in body weight for the 3 groups of sheep. Sheep in the PrC group showed the greatest increase in liver weights over the control. Liver weights were intermediate in sheep fed UPrC. These increased liver weights occurred partly from an accumulation of fat, of which a substantial portion was in the form of cholesterol.

When PrO was combined with the feeding of PrC in the second experiment, there was a 2-fold increase in the deposition of cholesterol (Table 2) and more severe cirrhosis of the liver than observed when PrC was fed alone in the first experiment (Table 1). While the 2 experiments are not strictly

TABLE 1

The Effect of Dietary Cholesterol on the Livers of Sheep Fed Protected (PrC) and Unprotected (UPrC) Cholesterol

	Control[a]	PrC[b]	UPrC[b]
Liver Weight (g)	530 ± 12.5	736 ± 30.4**	559 ± 30.7
Lipid content (g)	24.5 ± 0.2	97.6 ± 3.7**	48.9 ± 3.2**
Cholesterol content (g)	0.7 ± 0.02	21.1 ± 3.0***	8.1 ± 0.6
Initial body weight (kg)	42.9 ± 1.5	43.0 ± 0.3	43.7 ± 3.1
Body Weight gain (kg)	6.7 ± 0.3	5.3 ± 1.6	6.3 ± 0.3

Level of significance when tested for differences with the control group: *** 0.1%; ** 1%.
[a]Mean ± S.E., n=2.
[b]Mean ± S.E., n=3.

comparable, PrO feeding seems to facilitate a higher deposition of dietary cholesterol in the liver.

At the start of the second experiment, the plasma cholesterol concentrations were 74 ± 5.1 and 82 ± 3.0 mg % for the PrO + PrC and PrO groups, respectively. But, during the last 4 weeks of the experiment, the plasma cholesterol concentrations rose to 114.6 ± 2.5 and 127.0 ± 4.8 mg % for PrO + PrC and PrO groups, respectively, and were significantly different at the 5% level. Although the plasma cholesterol concentrations during the experimental period rose by 40.6 mg % in the PrO + PrC group compared with 45 mg % in the PrO group, the former group deposited 5 to 6 times more cholesterol in the liver (Table 2).

The ratio of cholesteryl ester to free cholesterol from lipids extracted from the livers of animals in the 2 experiments is given in Table 3. In both experiments, there was a marked increase in the proportion of cholesteryl esters in the PrC-fed groups. This increase was brought about by the deposition of cholesteryl esters as the amount of free cholesterol in relation to the total liver lipid showed little variation between treatments in both the experiments. The animals fed UPrC in the first experiment had liver cholesterol ratios (Table 3) and cholesterol contents (Table 1) that were similar to those of animals fed PrO in the second experiment, yet the former had livers that showed signs of cirrhosis and friability.

The fatty acid composition of cholesteryl esters in the 2 groups of sheep fed PrC and UPrC was very similar. Octadecenoates constituted nearly 45% of the total fatty acids in these groups; however, in the control group, they were only 23% of the total. The fatty acid composition of cholesteryl esters from the livers of sheep in the second experiment reflected the composition of PrO fed to these animals. Linoleic acid formed over 55% of the total fatty acids in both the PrO + PrC-fed and PrO-fed groups. These observations agree with the literature reviewed by Goodman (17) on the deposition of cholesteryl esters in monogastric animals. Goodman concluded that although liver cholesteryl esters can be changed by feeding polyunsaturated, lipid-rich diets, under normal feeding regimes the major cholesteryl esters that accumulate contain octadecenoates as the main fatty acids.

The work described here shows that hypercholesterolemia in sheep can result either from dietary cholesterol or in response to feeding lipid-rich diets. In experiments 1 and 2, when dietary cholesterol was fed in a protected form of at least 0.11% of the diet (1 g/day), it was readily absorbed and deposited in the liver, mostly in an esterified form, and resulted in cirrhosis. However, in experiment 2, in a treatment where no protected cholesterol was fed, hypocholesterolemia arose from cholesterolgenesis that occurred mainly in the epithelial cells of the small intestine in response to an

TABLE 2

The Effect of Dietary Protected Cholesterol (PrC) on the Livers of Sheep Supplemented with Protected Sunflower Oil (PrO)

	PrO	PrO + PrC
Liver weight (g)	547 ± 37.1	850 ± 104.1*
Lipid content (g)	37.4 ± 6.0	110.5 ± 26.7*
Cholesterol content (g)	8.0 ± 1.9	42.9 ± 12.0*
Initial body weight (kg)	30.7 ± 1.7	29.0 ± 0.3
Body weight gain (kg)	17.9 ± 1.0	17.7 ± 0.3

Level of significance when tested for differences with the PrO group: * 5%.
Mean ± S.E., n=3.

TABLE 3

The Effect of Dietary Protected (PrC) and Unprotected (UPrC) Cholesterol on the Ratio of Cholesteryl Ester to Free Cholesterol in the Liver Lipids of Sheep

	Ratio $\left(\frac{\text{cholesteryl ester}}{\text{free cholesterol}}\right)$
Experiment 1	
Control[a]	0.57 ± 0.03
UPrC[b]	2.46 ± 0.37
RpC[b]	8.54 ± 0.76**
Experiment 2	
PrO[b]	2.99 ± 0.49
PrO + PrC[b]	8.03 ± 0.73*

Level of significance when tested for differences with the control group for experiment 1 and the protected sunflower oil (PrO) group for experiment 2: ** 1%; * 5%.
[a]Mean ± S.E., n=2.
[b]Mean ± S.E., n=3.

increased lipid intake and assimilation of absorbed lipids (12). Only a small quantity of this endogenously derived cholesterol was deposited in the liver (Table 2). The metabolic processes and factors responsible for this difference in the fate of endogenously synthesized and dietary cholesterol are not clearly understood. However, during the absorption process a considerable role is played by the intestinal cells in controlling cholesterol metabolism through the formation and modification of lymph and plasma lipoproteins. These changes depend on the availability of cholesterol and bile salts as well as the nature of lipids absorbed from the digesta (28). The endogenous cholesterol derived from the bile and mucosal synthesis and incorporated into the intestinal lipoprotein remains much less esterified than the exogenous cholesterol of dietary origin, which is extensively esterified during absorption (13,29,30). Further indications that the endogenous and exogenous cholesterols in the lymph and plasma tend to form separate pools

and do not exchange freely (30-32) have been found, because these are either located at different sites on the same class of lipoproteins or are incorporated into different lipoprotein classes.

Compared with man and monogastric animals, a relative lack of information exists on plasma lipoprotein and dietary cholesterol metabolism in ruminants. In cattle, high-density lipoprotein (HDL) constitute over 80% and low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) 6% and 11%, respectively, of the total plasma lipoproteins (33). Like cattle, the major components of sheep plasma lipoprotein are HDL (70%) and LDL (17%) and reports disagree on the existence of VLDL (34,35).

Some researchers have postulated that protected cholesterol induces changes in sheep plasma lipoproteins that facilitate the uptake of cholesterol by hepatic cells. Low activity for lipogenesis and a lack of mechanism for mobilizing deposited cholesteryl esters from the liver could be contributing factors to an alteration in the plasma lipoprotein pattern. Reliable information on the activity of the hepatic enzyme lysozomal acid lipase (EC 3.1.1.13) in sheep liver is not available, but a deficiency of this enzyme in humans causes cholesteryl ester storage disease and Wolman's disease (36), which are very similar to the condition observed in sheep livers. Again, a similar disorder is observed in the livers of guinea pigs fed cottonseed oil + cholesterol (37,38) and is associated with the deficiency of the same enzyme.

This paper shows that the incorporation of low concentrations (0.11% to 0.22%) of protected cholesterol in the diets of sheep brings about marked changes in lipid metabolism and a massive deposit of cholesterol in the liver. Metabolic processes in these animals appear to be inadequate when compared with monogastric animals such as man who can cope with similar or even higher levels of cholesterol in their diets (30).

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REFERENCES

1. Palmquist, D.L. (1976) J. Dairy Sci. 59, 355-363.
2. McDonald, I.W., and Scott, T.W. (1977) in World Review of Nutrition and Dietetics (Bourne, G.H., ed.) Vol. 26, pp. 144-209, S. Karger, Basel.
3. Ashes, J.R., Gulati, S.K., Cook, L.J., Mills, S.C., and Scott, T.W. (1978) J. Lipid Res. 19, 244-249.
4. Wiggers, K.D., Jacobson, N.L., Getty, R., and Richard, M. (1973) Atherosclerosis 17, 281-295.
5. Barrows, K.K., Heeg, T.R., McGilliard, A.D., Richard, M.J., and Jacobson, N.L. (1980) J. Nutr. 110, 335-342.
6. Dietschy, J.M., and Wilson, J.D. (1968) J. Clin. Invest. 47, 166-174.
7. Dietschy, J.M., and Siperstein, M.D. (1967) J. Lipid Res. 8, 97-104.
8. Yeh, S.J., and Leveille, G. (1972) J. Nutr. 102, 349-358.
9. Dietschy, J.M., and Wilson, J.D. (1970) N. Eng. J. Med. 282, 1128-1138.
10. Thompson, J.R., Beitz, D.C., and Jacobson, N.L. (1977) J. Nutr. 107, 1632-1639.
11. Hanson, R.W., and Ballard, F.J. (1967) Biochem. J. 105, 529-536.
12. Nestel, P.J., Poyser, A., Hood, R.L., Mills, S.C., Willis, M.R., Cook, L.J., and Scott, T.W. (1978) J. Lipid Res. 19, 899-909.
13. Nestel, P.J., Havenstein, N., Scott, T.W., and Cook, L.J. (1974) Aust. N.Z. J. Med. 4, 497-501.
14. Avigan, J., and Steinberg, D. (1965) J. Clin. Invest. 44, 1845-1856.
15. Bieberdrof, F.A., and Wilson, J.D. (1965) J. Clin. Invest. 44, 1834-1844.
16. Grundy, S.M., and Ahrens, E.H., Jr. (1970) J. Clin. Invest. 49, 1135-1152.
17. Goodman, D.S. (1965) Physiol. Rev. 45, 747-839.
18. Wiggers, K.D., Jacobson, N.L., and Getty, R. (1971) Atherosclerosis 14, 379-389.
19. Jacobson, N.L., Richard, M., Berger, P.J., and Kluge, T.P. (1974) J. Nutr. 104, 573-579.
20. Wrenn, T.R., Bitman, J., Kahl, S., Weyant, J.R., and Wood, D.L. (1979) J. Dairy Sci. 62, 746-753.
21. Dougherty, K.A., Beitz, D.C., Richard, M.J., Sandry, P.B., and Jacobson, N.L. (1982) Nutr. Rpts. 25, 879-886.
22. Gulati, S.K., Cook, L.J., Ashes, J.R., and Scott, T.W. (1978) Lipids 13, 814-819.
23. Bligh, E.G., and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911-917.
24. Technicon Method Publication N-24A (1965) Technicon Corp., Ardsley (Chauncey), New York.
25. Scott, T.W., Setchell, B.P., and Bassett, J.M. (1967) Biochem. J. 104, 1040-1047.
26. Ashes, J.R., Haken, J.K., and Mills, S.C. (1980) J. Chromatogr. 187, 297-305.
27. Snedecor, G.W., and Cochran, W.G. (1980) Statistical Methods, 7th edn., Iowa State University Press, Ames.
28. Gangl, A., and Ockner, R.K. (1975) Gastroenterology 68, 167-186.
29. Sodhi, H.S., Orchard, R.C., Agnish, N.D., Varughese, P.V., and Kudchodkar, B.J. (1973) Atherosclerosis 17, 197-210.
30. Thomson, A.B.R., and Dietschy, J.M. (1981) in Physiology of the Gastrointestinal Tract (Johnson, L.R., ed.) Vol 2, pp. 1147-1220, Raven Press, New York.
31. Raphael, B.C., Patton, S., and McArthy, R.D. (1975) J. Dairy Sci. 58, 971-976.
32. Sodhi, H.S. (1967) in Proceedings of the 1967 Deuel Conference on Lipids - on the Fate of Dietary Lipids (Cogwill, G., and Kinsell, L.W., eds.) pp. 75-87, US Government Printing Office, Washington.
33. Ferreri, L.F., and Gleockler, D.H. (1979) J. Dairy Sci. 62, 1577-1582.
34. Nelson, G.J. (1973) Comp. Biochem. Physiol. 46B, 81-91.
35. Kubaseck, F.O.T., Leat, W.M.F., and Buttress, N. (1974) Proc. Nutr. Soc. 33, 47A-48A.
36. Burke, J.A., and Schubert, W.K. (1972) Science 176, 309-310.
37. Drevon, C.A., and Hovig, T. (1977) Acta Path. Microbiol. Scand. Sect. A. 85, 1-18.
38. Drevon, C.A. (1978) Atherosclerosis 30, 123-136.

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Physical State of Inhibitor Fatty Acids and Linoleate Solutions under Lipoxygenase Assay Conditions

MORTON J. GIBIAN* and **JOHN COLANDUONI**, *Department of Chemistry, Seton Hall University, South Orange, NJ 07079*

ABSTRACT

A variety of fatty acids, which are potential competitive inhibitors of soybean lipoxygenase (EC 1.13.11.12), give kinetically unstable mixtures in standard assay solutions containing linoleate (substrate). In the assay solution (pH 10, 0.1 M borate, 1.63% ethanol), each fatty acid by itself shows normal surface tension vs concentration behavior; but, despite a range of solubilization techniques in the presence of 10 μM or higher linoleate and low concentrations of these materials, irreproducible surface-tension readings and inhibition kinetics result. This inhomogeneity (or kinetic instability) disappears as the concentration increases. Critical micelle concentration (CMC) values of mixtures are not additive, and binary mixture behavior depends on fatty acid structure. Several lines of observation, including CMC values and actual surface tension (γ) values for several systems, suggest premicellar heterodimer or higher mixed aggregate formation. Lipids with K_i significantly above the irreproducible surface-tension range give good kinetic behavior, and K_i is reported. The results are in accord with earlier work on aspects of these systems. Complementary solution physical studies must be done for any kinetic (or specificity) determinations of enzymes using lipids.

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INTRODUCTION

Soybean lipoxygenase (E.C. 1.13.11.12) catalyzes the reaction of *cis,cis*-1,4-pentadienyl fatty acids (linoleate is a typical substrate) with molecular oxygen to form the conjugated *trans, cis*-dienyl allylic hydroperoxides. As an approach to examination of interactions at the active site of this enzyme, we decided to investigate nonsubstrate fatty acids as potential competitive inhibitors to measure binding (K_i) as a function of structure (chain length, unsaturation, and so forth). The literature contains no rigorous data of this kind for lipoxygenase, but some inhibition studies are available at one substrate concentration or with nondefined kinetics. Advances in understanding the steady-state kinetics of this enzyme should have now made an analytical approach possible here (1-5).

However, very early in the work we found that binary mixtures of linoleate and many candidate inhibitor fatty acids (below the reported critical micelle concentration (CMC) values of both components) do not show reproducible kinetic behavior despite careful and persistent attempts to normalize procedures for making up and mixing solutions (vide infra). These results led us to examine the physical state of the solution by surface tension measurements—the primary subject of this report.

Other reasons why a physical-state study is now appropriate follow. Researchers have observed, in many previous studies, that Lineweaver-Burk plots for lipoxygenase-catalyzed oxygenation of linoleic acid show significant apparent substrate inhibition at high S, often attributed to linoleate micelle formation. We shall soon report a detailed examination of the turnover kinetics with emphasis on the behavior at high S (and the effect of product there); in the current context, however, the study of solution properties is of the utmost importance. Irregular behavior with varying organic solvents had also been reported earlier, although with low constant ethanol this problem is largely prevented. The effect of pH on the enzymic process has been partially ascribed to the physical effects of the medium.

The present study, as a step toward defining solution properties of these fatty acid mixtures, is essential to sorting out these varied phenomena.

*To whom correspondence should be addressed.

The notation for fatty acids follows the system in which a,b-x:y indicates a fatty acid of x carbons with y *cis* double bonds at positions a and b from the CO_2H; *trans* olefin is designated by (t) following the position number; $SC_{10}S$ and $SC_{12}S$ are sodium decyl and sodium dodecyl sulfates; 9,12(OH)-18:1 is ricinoleic acid; CMC is critical micelle concentration. Surface tension is γ.

MATERIALS AND METHODS

Lipoxygenase

Commercial soybean enzyme (from Sigma Chemical Co., St. Louis, MO, ca. 150,000 EU/mg, usually 15-17 μmols/min/mg E) was chromatographed on DEAE Sephacel (ca.

2.6 x 30 cm column for 500 mg) using a 0.01-0.2 M linear gradient of phosphate buffer (pH 6.8) containing 0.1 mM EDTA. Resultant lipoxygenase-1 (pure by disc gel electrophoresis, typical specific activity in our assay of ca. 100 μmols/min/mg E) lost little activity for up to 6 months at 0-4 C at ca. 0.5 mg/ml (mg protein = 0.7 A_{280}) in the eluant buffer.

Fatty Acids

Most of the fatty acids were from Sigma Chemical Co., the rest from Nu-Chek-Prep. Inc., Elysian, MN (all >99% pure). Except for a few of the very expensive ones (surface tension work is not reported for these), fatty acids were purified on silica gel using hexane/ether/acetic acid (65:30:1) as eluant, and purity was checked by thin layer chromatography (TLC). They were stored neat at −10 C under argon. Stock solutions were made in ethanol and kept anaerobic until used. Linoleyl hydroperoxide was synthesized enzymatically and purified as previously described (3).

Methods

All kinetic and surface tension experiments were carried out in pH 10 (0.1 M) borate buffer containing (after all additions) 1.63% ethanol. Water was double distilled; buffer components were the best grades available. Assays were at 25 C in a Cary 219 spectrophotometer at 234 nm in air-saturated buffer (250 μM O_2); they contained 0.5 μM product hydroperoxide, 5 to 50 μM linoleate, other fatty acid as indicated, and were initiated by an aliquot of enzyme to produce ca. 3×10^{-4} mg/ml final lipoxygenase concentration in the cell. Where K_i values are reported in Table 1, double reciprical plots for a wide range of linoleate were done vs at least 4 inhibitor concentrations (duplicate runs) and then the slope data was plotted vs (I) in the usual manner. The data for those materials are in accord with competitive inhibition.

Surface tension experiments were with a Fisher Model 20 Surface Tensiometer (duNuoy platinum-iridium ring). Except where noted, readings were taken for 20 min after initial mixing.

RESULTS

Qualitative Observations

Reproducible and rational competitive inhibition kinetics are obtained for 9-18:1, 9-16:1, 9,12(OH)-18:1, $SC_{10}S$ and $SC_{12}S$ over good concentration ranges of both inhibitor (up to 0.8 mM) and linoleate in pH 10 borate buffer (0.1 M, 1.63% ethanol). Under the same conditions, at 20 μM linoleate (ca. K_m for lipoxygenase), highly irreproducible results are obtained for 0.01 to 1.0 mM 10:0, 12:0, 14:0, 18:0, 9(t)-18:1, and 9(t),12(t)-18:2. While 18:0 and 9(t)-18:1 give visible precipitates, the others appear clear. These latter fatty acids are well below their reported solubilities and CMC. Further attempts to obtain homogeneous and reproducibly behaving solutions, primarily with 0.1 mM 10:0 and 12:0 in the presence of 20 μM linoleate, are outlined below. The criterion used in this part of the study is reproducible kinetic behavior. (a) Shaking the complete assay mixture minus enzyme for up to 15 min before measurement does not improve reproducibility. (b) Vigorous vortex mixing of the fatty acids in the assay solution for up to 25 min was tried. The kinetic results were nonuniform and depended very strongly on the

TABLE 1

Critical Micelle Concentrations and Binding Properties[a]

Fatty acid	CMC (mM)		K_i (mM)
	Alone	+10 μM linoleate	
9,12-18:2 (linoleate, substrate)	0.096	–	(K_m = 0.018)
10:0	41	ca. 70	NA[b]
12:0	9	ca. 20	NA[b]
9-16:1	–	–	0.12
9-18:1	0.03	0.07	0.15
9(t),12(t)-18:2	0.3	NA[b]	–
9,12(OH)-18:1	ca. 45	–	0.29
$SC_{10}S$	10	ca. 10[c]	–
$SC_{12}S$	0.7	0.9	0.4

[a]Conditions as described in text.

[b]Solubility problems preclude obtaining values.

[c]A discontinuity occurs at 0.1 mM, then a second one at 10 mM.

interval (up to 15 min) between vortexing and initiation of reaction (adding enzyme). Often no inhibition was observed. (c) When the sodium salt of the fatty acid is made beforehand and then added to the assay mixture, no improvement is observed (it is often worse, especially with 9(t)-18:1). (d) Assays run in pH 10 glycine and carbonate buffers and in distilled water, rather than borate, show disparate results. (e) Varying the ethanol content from 0.8 to 3.3% does not improve the assays. In 3.3% EtOH, even 1 mM 10:0 and 12:0 show no inhibition. (f) When lauric acid (12:0) is deposited in a flask in either chloroform or ethanol (separate experiments), the solvent then removed while the flask is rotated, and then buffer added with vigorous shaking, the data are still inconsistent. (g) Even when the mixtures of b, c, and f above (vortexed acids, salts and deposited acids) are subjected to up to an hour of ultrasonication with mild warming, no better consistency results.

The inescapable conclusion is that these are inhomogeneous mixtures. Direct physical behavior studies are described in the next section.

Surface Tension Measurements

By themselves, all the fatty acids examined behave "well" (reproducible results, stable solutions) in surface tension determinations in the standard assay mixture including the 1.63% ethanol at 24 C. Although ethanol could cause some problems in these measurements, the solutions were treated identically throughout, only relative behavior is being addressed, and it was most important that all studies be under assay conditions. Representative data for surface tension (γ) as a function of the logarithm of concentration for linoleate (9,12-18:2), 9-18:1, $SC_{12}S$, and 12:0 alone are shown in Figure 1. Other acids, including 10:0, 9(t)-18:1, 9(t), 12(t)-18:2, 9-16:1, and 9,12(OH)-18:1 as well as $SC_{10}S$ also give good plots. Stearic acid (18:0), however, is too insoluble.

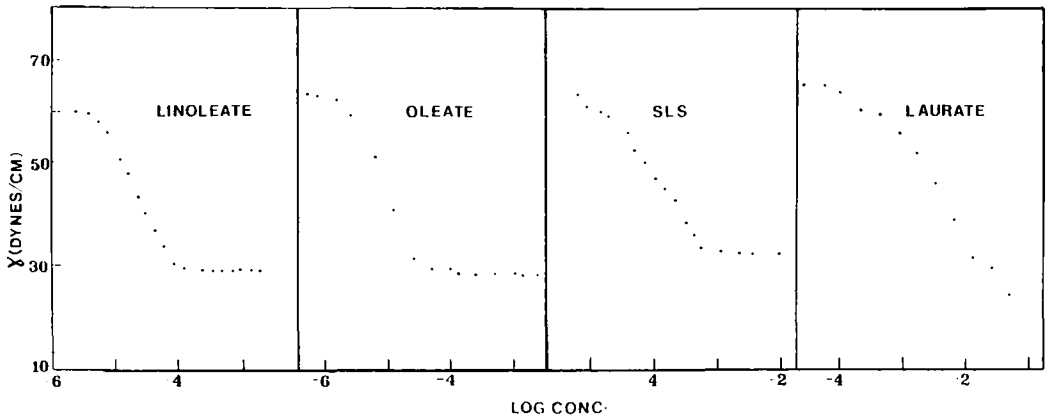

FIG. 1. Surface tension of individual fatty acids as a function of the logarithm of their concentrations in assay solution as described in the text (0.1 M pH 10 borate, 1.63% ethanol).

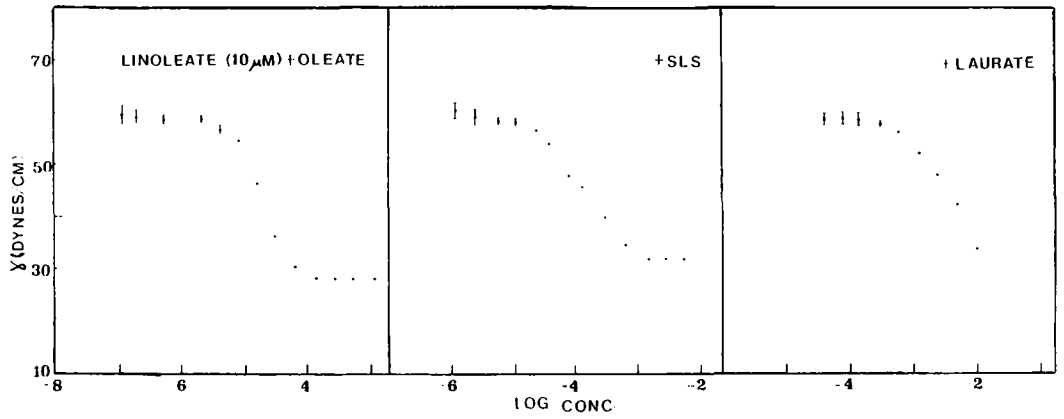

FIG. 2. Surface tension of solutions at constant 10 μM linoleate as oleate, $SC_{12}S$, and laurate are varied (0.1 M borate, pH 10, 1.63% ethanol).

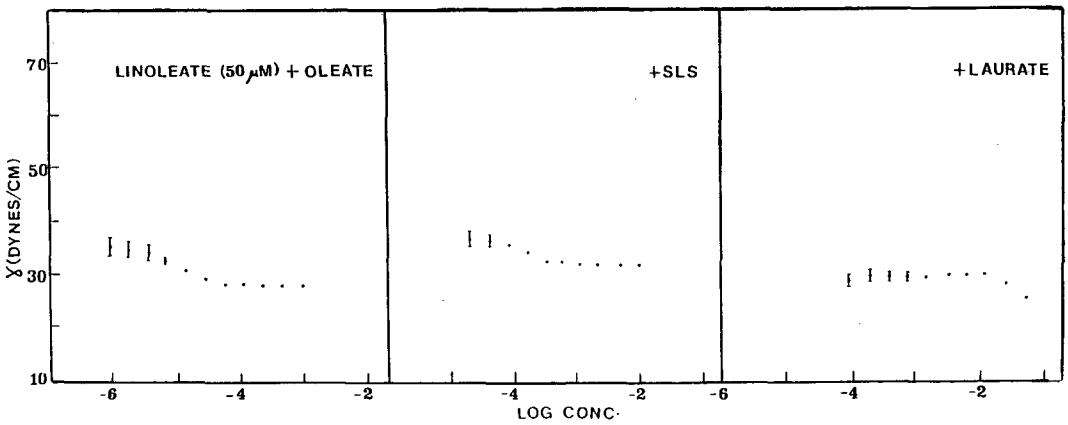

FIG. 3. As for Figure 2, but with 50 μM constant linoleate.

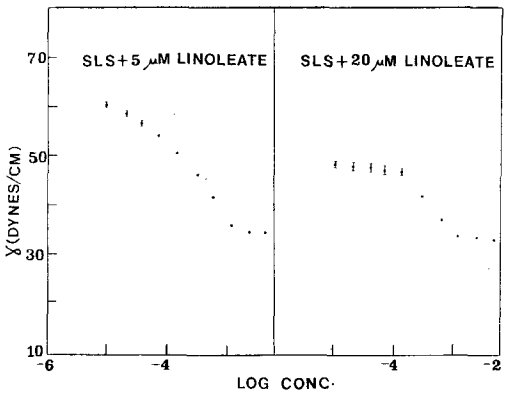

FIG. 4. Conditions as in other figures, but with varying $SC_{12}S$ at constant 5 μM or 20 μM linoleate.

When binary mixtures including fixed levels of linoleate (lipoxygenase substrate) together with varying concentrations of the other fatty acids are examined, however, the underlying problem appears. Data were obtained over a fairly wide range; those at relatively low and relatively high linoleate will be presented. Figure 2 shows surface tension data for varying concentrations of 9-18:1, $SC_{12}S$, and 12:0 in the presence of constant 10 μM linoleate; Figure 3 has the graphs for the same materials at 50 μM linoleate. Data for $SC_{12}S$ is also shown at both lower and intermediate (5 and 20 μM) linoleate concentrations (Fig. 4). In all graphs, the experimental error (reproducibility, spread of values or the stability of the surface tension reading) is indicated by a bar for those cases where this value is larger than the point shown. For the determinations with large error bars, the values do not stabilize for at least 20 min. Note that the CMC of linoleate is ca. 100 μM, above all experiments. The time dependence was also reported by Lagocki et al. (5).

The common feature of these surface tension experiments for both those fatty acids that give good inhibition kinetics and those that do not is that alone all exhibit smooth plots and stable readings, but with linoleate present (at all substrate concentrations, clearly worse as concentration increases) there is a region below the CMC of either component in which surface tension readings are unstable. Near and above the CMC, well-defined surface tension values are observed. Indeed, those fatty acids that give reproducible inhibition kinetics do so only because the K_i is outside the concentration region in which solutions behave poorly. These experiments were repeated many times, with different ways of mixing and waiting various time intervals before taking measurements.

Mixtures of the above materials and linoleate with and without added enzymic product (ca. 0.1 μM) behave in very much the same way. Surface tension determinations were also performed at several constant product concentrations vs varying linoleate. The results are that the CMC of linoleate (97 μM alone) is unaffected by 0.1 μM product, but increases slightly to 100 μM at 5 μM product, and to 150 μM at 50 μM product.

Another aspect is that all the CMC values in the presence of 10 μM linoleate are higher than those for the fatty acid alone, opposite to the result expected if effects were additive. For several fatty acids ($SC_{12}S$ data are shown), the CMC is irregular as linoleate is regularly increased. Both this and the low concentration instabilities suggest specific interactions between the individual fatty acid and linoleate.

Lipoxygenase Competitive Inhibitions

Instabilities of solutions used for inhibition studies are also manifest in direct observation of solution properties. The unstable concentration range for many of these materials turns out to be in the region at which one must have inhibition data (around the extrapolated K_i) in order to analytically prove competitive behavior and determine accurate binding constants. For those compounds that can be clearly shown to be competitive, the values of K_i along with CMC are reported in Table 1. No apparent correspondence was found between CMC and K_i for these fatty acids.

DISCUSSION

The physical state of substrate solutions has been a long-standing difficulty in determining reaction kinetics of many lipid-using enzymes, and lipoxygenase is no exception. Attempts to circumvent this problem have often led to a new set of intricacies. For example, adding water-miscible organic solvent in high quantities (greater than ca. 3%) or adding detergents (Tween and others) may indeed produce homogeneous solutions, but also commonly leads to inhibition by competing for a hydrophobic site on the enzyme or by affecting its conformation (1-3,6,7).

The research reported here shows that, under optimal assay conditions for lipoxygenase, almost all fatty acids investigated behave well alone in solution, but mixtures with linoleate show inhomogeneity at low concentrations. Manifest in significant instability in the surface tension readings, the effect disappears as the fatty acid concentration approaches the CMC. For many of the potential inhibitors, this occurs in the range of the extrapolated K_i; therefore, obtaining good data for competitive inhibition is impossible. CMC values for the fatty acids studied do not show a regular pattern of dependence as a second fatty acid (linoleate here) is added. These observations indicate specific interactions between pairs of these molecules that are dependent on chain length and unsaturation.

The surface-tension plots of the fatty acids alone give results that are in reasonable agreement with literature values of γ, general behavior of the curves, and CMC values (8-10).

Irreproducible behavior of γ at certain concentrations could be caused by premicellar aggregate formation (11,12), although the reason that it is specific for mixtures of fatty acids is not at all clear. That this behavior is not just a surface effect is shown by the corresponding problems in bulk kinetic studies in the lipoxygenase work. We have not altered the pH above 10 for enzyme stability reasons, and lower pH increases the problem of acid-salt associations (8). Apparently the associative or insolubility phenomenon that occurs at intermediate (or low) concentrations of mixtures is overcome when enough hydrophobic material is in solution.

The data also suggest some possible quantitative conclusions. The CMC of solutions with varying linoleate at several constant product concentrations shows an increase in CMC equal to the concentration of product. This suggests a heterodimer between linoleate and its 13-hydroperoxide that decreases the free linoleate by an amount equal to the product concentration. The limiting γ of the 3 materials shown in Figure 2 (and for decanoate as well) at low concentration is ca. 59 dyne/cm, whereas the γ for 10 μM linoleate is 53 dyne/cm (γ of buffer with ethanol alone is 65 dyne/cm). These experiments were repeated numerous times, and the difference is well outside experimental error under our conditions of measurement. This is as if free linoleate has been removed from solution. For 10 μM oleate (Fig. 1), γ is 44 dyne/cm, while in Figure 2, 51 dyne/cm (10 μM linoleate) represents much less oleate, again, as if free oleate has been removed from solution. All the binary mixtures, including that of product with linoleate in both our data and those obtained in a somewhat different manner by Verhagen et al. (8), show an increase in CMC, not the decrease expected on the basis of classical theory. These observations, together with several points made above, strongly support the conclusion that heterodimers and higher heteroaggregates (but significantly smaller than micelles) are formed in these mixtures.

Physical Studies

Earlier physical studies concerning fatty acids in aqueous solution show many of the complexities encountered here. Although many workers prefer to consider plots of γ vs log c (or other physical property vs appropriate function of concentration) accurate representations of monomer-micelle 2-state equilibria, this clearly often does not represent physical reality.

Mysels and Florence (13), in a review, concluded that fatty acids in water are essentially always polydisperse, and mixtures lead to much more complexity. Profound effects are noted for charged materials (which can give precipitates that then dissolve at higher concentrations of detergent), and for trace impurities (which can give lower γ values below the CMC, followed by solubilization in aggregates to give

higher values again--leading to minima in the plots).

Careful evaluation (13) of the γ data from several studies of sodium dodecyl sulfate ($SC_{12}S$) showed considerable scatter that could not be the result of surface tension errors (easily reproducible to ±0.1 dyne/cm). Small amounts of impurity often led to minima, although great care was taken to purify the $SC_{12}S$. Even the purest material showed a dynamic surface tension effect in the concentration range used in the present work; rapid expansion of the surface resulted in a slow (by seconds, minutes or hours) return to equilibrium.

Earlier, Shedlovsky et al. (14) had seen aging effects in physical studies of those materials—often taking 30 min (but sometimes up to 20 hr) for equilibration after mixing of lipids. Other early work had similar observations (10,15).

Lucassen (16) and Eagland and Franks (17), both in the mid-1960's, examined the concentration dependence of the pH of fatty acids in water. Apparently traces of CO_2 cause turbidity at low substrate concentrations. Evidence also exists from conductance and infrared studies for acid dimers, acid-soap dimers and possibly for other aggregates. Again, kinetic factors are obviously at play (slow approach to equilibria).

Converging opinion from these and other investigations is that premicellar aggregates are important in many (or most) systems (11-13, 18). The sum of these studies makes our results quite credible. The problem is that this behavior has usually either not been recognized or been ignored by lipid enzymologists.

Enzyme Studies

Fairly recent studies have addressed the state of the substrate in lipoxygenase assays. Bild et al. (19) show that at high concentration (0.1 M), the apparent titrimetric pK_a of linoleic acid is ca. 7.9. A shorter chain acid shows a normal pK_a of ca. 4.9, and although the original workers do not draw any general conclusion or examine a variety of examples, that this is a micelle effect seems likely (the effect is certainly in the expected direction, carboxylic acid ionization should be retarded in more hydrophobic or anionic surroundings). Linoleyl sulfate showed a not dissimilar effect.

Second, Verhagen et al. (8) studied the behavior of linoleate and 13-hydroperoxy-9,11(t)-octadecadienoate (the lipoxygenase product) in assay-type media. At pH 10, the CMC for linoleate was 0.167 mM, whereas at pH 8 and 9 the CMC was lower (<0.1 mM) and the log c vs γ curve indicates a good deal of acid-anion dimer formation. A state diagram was constructed for linoleate and product mixtures in terms of monomers, micelles, and mixed acid-anion dimers. The quantitative and qualitative results in our study agree with the Verhagen work (8) in terms of γ values and the location of the dimer-forming region. They did not add other fatty acids.

Finally, Gatt and Bartfai (20) have examined formal kinetic models for enzyme reactions of micelle-forming substrates. The approach was based on a 2-state model (monomer or micelle), with different V_{max} and K_m for each, that should be considered for any lipid-using enzyme system. However, the irregularities reported here fall outside the Gatt treatment. Whereas lipoxygenase kinetic behavior resembles that predicted for certain of these formalisms, earlier work, along with recent results in our laboratory (manuscript in preparation), shows that the high substrate behavior and enzyme concentration dependence do not have their origin in physical substrate problems. Before the formal Gatt treatment may be applied, however, examining the particular system for physical behavior is essential to ascertain what a nominal S_o is measuring.

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We appreciate the suggestion and analysis by a referee that these data quantitatively support the heterodimer concept.

REFERENCES

1. Vliegenthart, J.F.G., and Veldink, G.A. (1982) in Free Radicals in Biology (Pryor, W.A., ed.) Vol. V, Chapter 2, Academic Press, NY.
2. Gibian, M.J., and Galaway, R.G. (1977) in Bioorganic Chemistry (Van Tamelen, E.E., ed.) pp. 117-136, Academic Press, NY.
3. Gibian, M.J., and Galaway, R.G. (1976) Biochemistry 15, 4209-4214.
4. Egmond, M.R., Brunori, M., and Fasella, P.M. (1976) Eur. J. Biochem. 61, 93-100.
5. Lagocki, J.W., Emken, E.A., Law, J.H., and Kézdy, F.J. (1976), J. Biol. Chem. 251, 6001-6006.
6. Allen, J.C. (1968) Eur. J. Biochem. 4, 201-208.
7. Galpin, J.R., and Allen, J.C. (1977) Biochim. Biophys. Acta 488, 392-401.
8. Verhagen, J., Vliegenthart, J.F.G., and Boldingh,

J. (1978) Chem. Phys. Lipids 22, 255-259.
9. Fendler, J., and Fendler, E. (1975) in Catalysis in Micellar and Macromolecular Systems, pp. 26 and 27, Academic Press, New York.
10. Murkerjee, P., and Mysels, K.J. (1971) in Critical Micelle Concentrations of Aqueous Surfactant Systems, Nat. Bur. Stand., Washington, DC, pp. 14-17.
11. Menger, F.M. (1979) Acc. Chem. Res. 4, 111-117.
12. Mittal, K.L. and Fendler, E.J. (1982) Solution Behavior of Surfactants, Plenum Press, New York.
13. Mysels, K.J., and Florence, A.T. (1973) J. Colloid Interfac. Sci. 43, 577-582.
14. Shedlovsky, L., Ross, J., and Jakob, C.W. (1949) J. Colloid Sci. 4, 25-33.
15. Herzfeld, S.H., Corrin, M.L., and Harkins, W.D. (1950) J. Phys. Colloid Chem. 54, 271-282.
16. Lucassen, J. (1966) J. Phys. Chem. 70, 1824-1830.
17. Eagland, D., and Franks, F. (1965) Faraday Soc. Trans. 6, 2468-2477.
18. Tanford, C. (1980) The Hydrophobic Effect, 2nd Ed., pp. 63ff, J. Wiley & Sons, New York.
19. Bild, G.S., Ramados, C.S., and Axelrod, B. (1977) Lipids 12, 732-735.
20. Gatt, S., and Bartfai, T. (1977) Biochim. Biophys. Acta 488, 1-12.

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Lipid Composition of Liver Mitochondria and Microsomes in Hyperthyroid Rats

F.M. RUGGIERO[a], C. LANDRISCINA[b], G.V. GNONI[a] and E. QUAGLIARIELLO[a],
[a]Research Unit on Mitochondria and Energy Metabolism, C.N.R., Via G. Amendola 165/A, 70126 Bari and [b]Biochemistry Laboratory, Veterinary Faculty, University of Bari, Bari, Italy[1]

ABSTRACT

Triiodothyronine-induced alteration of the lipid pattern in rat-liver mitochondria and microsomes has been investigated. In mitochondria, a 25% total cholesterol decrease and a 14% phospholipid increase have been detected. In these hyperthyroid rat liver organelles, a strong decrease in the total cholesterol/phospholipid molar ratio occurs. On the contrary, in microsomes from the same animals, a decrease of about 23% has been measured for both total cholesterol and phospholipids; hence, in this fraction, the total cholesterol/phospholipid molar ratio is unaffected by hyperthyroidism. The liver mitochondrial phospholipid composition, unlike the microsomal composition, is altered significantly in hyperthyroid rats; a 7.4% phosphatidylcholine decrease is accompanied by a similar additive percentage increase of both phosphatidylethanolamine and cardiolipin. In regard to total phospholipid fatty acid composition in liver microsomes from hyperthyroid rats, no variation has been observed compared with the control rats, whereas in mitochondria from the same animals, a meaningful linoleic acid decrease with a similar arachidonic acid increase has been found. In addition to fatty acid alteration, the separated mitochondrial phospholipid classes also exhibit some increase in stearic acid. Among phospholipids, cardiolipin changes the most of the esterified fatty acids in hyperthyroid rat liver. In this compound, a strong increase in the percentage of both palmitic and stearic acid and a 32.4% decrease of linoleic acid have been found.
Lipids 19:171-178, 1984.

INTRODUCTION

Cholesterol and phospholipids are important constituents of biological membranes and a cholesterol/phospholipid ratio peculiar to particular membranes can be detected (1). Besides membrane fluidity, membrane lipid composition also influences the associated enzyme activity (2). Among the parameters shown to affect biomembrane dependence are cholesterol/phospholipid molar ratio (3), phospholipid composition (4), degree of fatty acyl unsaturation (2) and lipid/protein ratio (5).

Several studies have indicated that enzymes influenced by thyroid hormones are generally those associated with membranes (6-9). In addition to their effects on intracellular mechanism, these hormones have been found to influence mainly membrane fluidity; hence, all other metabolic effects seem to be secondary to this action (10,11).

The purpose of the present work was to control the alteration induced by hyperthyroidism on the lipid pattern in rat-liver mitochondria and microsomes. This would give further support to the reasons for triiodothyronine-induced alteration of several enzyme activities, e.g., those related to substrate oxidation (12), electron transport chain (13), swelling (3) and other metabolic processes (7,8).

The data obtained indicate that in liver mitochondria from hyperthyroid rats, the decrease of cholesterol content, accompanied by a small increase of phospholipids, leads to a reduction in the total cholesterol/phospholipid molar ratio. In these particles, a meaningful alteration of phospholipid composition and an increase of arachidonic acid in phosphatidylcholine and phosphatidylethanolamine as a consequence of the hyperthyroid state has also been noticed. In particular, the cardiolipin fatty acid pattern shows the greatest alteration. In microsomes from hyperthyroid rats, however, a similar decrease of both cholesterol and phospholipids occurs whereas the pattern of the latter compounds and their esterified fatty acids is slightly affected.

EXPERIMENTAL PROCEDURE

Animals

Throughout these studies 30-day-old male Wistar rats fed ad libitum with a standard diet were used. Animals were made hyperthyroid as previously described (14). 3,3',5-L-Triiodothyronine (30 μg/100 g body wt), dissolved in 0.9% NaCl-propyleneglycol (40:60, v/v), was injected intraperitoneally by a single daily injection for 5 consecutive days. Control animals

[1]Correspondance should be addressed to: Centro di Studio sui Mitocondri, e Metabolismo Energetico, c/o Università di Bari, Via G. Amendola 165/A, 70126 Bari, Italy.

received only the solvent, for the same period, in the same way. The drug dose and rat treatment duration were chosen to obtain a variation of the haematic triiodothyronine level without significantly changing the body weight of the animals (15). During the treatment period, the body weight increase of T_3-treated animals was essentially the same as that of the control rats (3.0-3.2 g/day/rat) (16). The daily food intake was the same for each treated or untreated animal (12). A significant variation in the whole liver phospholipid content of hyperthyroid rats was observed. After 5 consecutive days of T_3 administration, the whole liver phospholipid phosphorus content in these animals increased by 27% (from 26.8 μmol phospholipid Pi/g tissue in euthyroid to 34.1 μmol phospholipid Pi/g tissue in hyperthyroid rats). This was probably caused by stimulated phospholipid synthesis (17). Animals were killed 24 hr after final administration. Liver subcellular fractions were obtained as previously described (12). Mitochondria and microsomes were suspended in 0.25 M sucrose and protein was determined by the Lowry method (18).

High Pressure Liquid Chromatography (HPLC) Analysis of Cholesterol, Phospholipids and Fatty Acids

Cholesterol, phospholipids and fatty acids were analyzed by HPLC, using a Beckman 344 gradient liquid chromatograph equipped with a Perkin-Elmer LC-55B spectrophotometric detector.

To determine cholesterol, the chromatographic column was Altex ultrasphere-ODS, reverse phase (4.6 × 250 mm) from Beckman Instruments, Palo Alto, CA. First, mitochondria or microsomes (10 mg protein) were saponified with alcoholic KOH for 60 min at 45 C and extracted with hexane, then the extract was evaporated and the residue dissolved in 2-propanol, an aliquot of which was injected into the column. The mobile phase was 2-propanol/acetonitrile (50:50, v/v) at a flow rate of 1 ml/min (19).

Total lipids were extracted from mitochondria or microsomes (30-40 mg protein) with chloroform/methanol (1:1, by vol) by the Bligh and Dyer procedure (20). A portion of this extract was digested at 180 C with perchloric acid, then the phospholipid phosphorus content was determined by the Nakamura method (21). Phospholipids were separated by an Altex ultrasil-Si column (4.6 × 250 mm) from Beckman Instruments. The chromatographic system was programmed for gradient elution using mobile phases: solvent A, hexane-2-propanol (6:8, v/v) and solvent B, hexane-2-propanol-water (6:8:1.4, v/v/v). The percentage of solvent B in solvent A was increased in 15 min from 0% to 100%. Flow rate was 2 ml/min and detection at 206 nm. Quantitative estimation of single phospholipid species was done by comparing the calculated peak areas with those of each phospholipid standard solution.

To analyze fatty acids, total or single phospholipids [phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and cardiolipin (DPG)] were collected after elution from the ultrasil-Si column and saponified with 5% KOH in 50% aqueous methanol for 40 min at 90 C. After acidification, the solution was first extracted with chloroform, next taken to dryness and then esterified with m-methoxyphenacyl bromide for HPLC analysis (22). For this analysis, the column was an Altex ultrasphere-ODS reverse phase (4.6 × 250 mm); the mobile phase was tetrahydrofuran/aetonitrile/water (45:25:35, v/v/v) at a flow rate of 2 ml/min. To estimate single fatty acid concentration, the calculated peak areas were compared with those of each fatty acid standard solution (22).

RESULTS

The effect of triiodothyronine-induced hyperthyroidism on rat liver mitochondrial, microsomal phospholipid and total cholesterol content is shown in Table 1. From this table, total cholesterol can be seen to decrease by 25% and, inversely, phospholipids increase by ca. 14% in mitochondria. Consequently, the inverse relationship between the change in total cholesterol and phospholipid content observed in these organelles causes a 40% decrease in the total cholesterol/phospholipid molar ratio. This table also shows that in microsomes, from the same rats, both total cholesterol and phospholipids decrease in the same amount, 23%. Hence, in this subecllular fraction, the total cholesterol/phospholipid molar ratio remains unaffected by hyperthyroidism.

Phospholipid composition of liver mitochondria is significantly different in eu- and hyperthyroid rats, especially the amount of phosphatidylcholine, phosphatidylethanolamine and cardiolipin. While the first compound decrease by 7.4%, the other 2 increase by 4.2 and 4.6%, as shown in Table 2. On the contrary, no appreciable variation in microsomal phospholipid composition of both types of animals occurs except for phosphatidylethanolamine, which increases slightly (3.8%) in hyperthyroid rats.

A typical mitochondrial (A) or microsomal (B) phospholipid separation carried out by our modified HPLC method is reported in Figure 1. The order of elution in this system is NL (as a

TABLE 1

Effect of Triiodothyronine Administration on Total Cholesterol and Phospholipid Content in Rat Liver Mitochondria and Microsomes

	Mitochondria[a]		Microsomes[a]	
	Euthyroid	Hyperthyroid	Euthyroid	Hyperthyroid
Total cholesterol	16 ± 2	12 ± 2[b]	73 ± 9	56 ± 6[b]
Phospholipids	166 ± 10	189 ± 12[b]	650 ± 50	500 ± 60[c]
Ratio cholesterol/ phospholipid	0.1 ± 0.01	0.06 ± 0.008[c]	0.11 ± 0.009	0.11 ± 0.01

[a]Each value represents the mean obtained for 7 experiments with 8 rats each ± SE. Values are expressed as nmol/mg protein.
[b]$p < 0.01$ vs euthyroid.
[c]$p < 0.001$ vs euthyroid.

TABLE 2

Phospholipid Composition in Rat Liver Mitochondria and Microsomes as Determined by HPLC

	Distribution (mol %)[a]			
	Mitochondria		Microsomes	
Phospholipid	Euthyroid	Hyperthyroid	Euthyroid	Hyperthyroid
PC	49.1 ± 2.1	41.7 ± 2.5[b]	65.5 ± 2.0	62.7 ± 1.9
PE	27.3 ± 1.6	31.5 ± 1.5[b]	19.0 ± 1.0	22.8 ± 1.2[b]
DPG	13.4 ± 1.0	18.0 ± 1.3[c]	1.8 ± 0.6	2.0 ± 0.8
PS	1.0 ± 0.2	0.8 ± 0.3	3.0 ± 0.7	2.4 ± 0.5
PI	6.8 ± 1.4	6.0 ± 0.9	8.7 ± 0.9	8.6 ± 1.0
SPH	2.4 ± 0.5	2.0 ± 0.7	2.0 ± 0.5	1.5 ± 0.6

[a]Each value represents the mean obtained for 7 experiments with 8 rats each ± SE.
[b]$p < 0.01$ vs euthyroid.
[c]$p < 0.001$ vs euthyroid.

single peak), DPG, PE, PI, PS, PC and SPH. The gradient seapration we used has been found to be successful in separating 6 phospholipid components.

Several studies have proven that thyroid hormones can modulate membrane fluidity by changing the fatty acids of their lipid components (23-25). This aspect has been further investigated in our laboratory and, in Table 3, liver mitochondrial composition of total and single phospholipid fatty acids from eu- and hyperthyroid rats is reported. The mitochondrial fatty acid pattern in total and single phospholipids changes significantly in hyperthyroid rats compared with control rats. An important common variation regards the 18:2 content, which decreases significantly in favor of 20:4, whose amount increases by almost the same percentage. The analysis of individual phospholipids shows that in each compound, even the percentage of 18:0 is significantly higher in hyperthyroid rats. In particular, cardiolipin fatty acids show a remarkable alteration; here palmitic and stearic acids increase by 21.5% and 9.2%, respectively, whereas linoleic acid decreases by 32.4%. The last 2 lines of this table show that in liver mitochondria from hyperthyroid rats, the unsaturation index decreases significantly only in phosphatidylinositol and cardiolipin, whereas the desaturating activity, measured as the ratio of the unsaturated fatty acids, 20:4/18:2, is well above the control value in all the phospholipid species. Microsome phospholipid fatty acids undergo no significant change in hyperthyroid rats, as shown in Table 4. The desaturation index and 20:4/18:2 ratio are almost the same as those of the control rats.

DISCUSSION

Several studies have shown that many membrane-associated enzymes, e.g., succinate dehydrogenase, cytochrome c oxidase (6), adenine nucleotide translocase (7), (Na^+-K^+)-ATPase (8) and those responsible for protein synthesis (9), are influenced by thyroid hormones. Perviously, we have reported that mitochondria from hyperthyroid rats exhibit a noticeable increase in oxygen consumption when succinate, NADH, palmitate or malate are

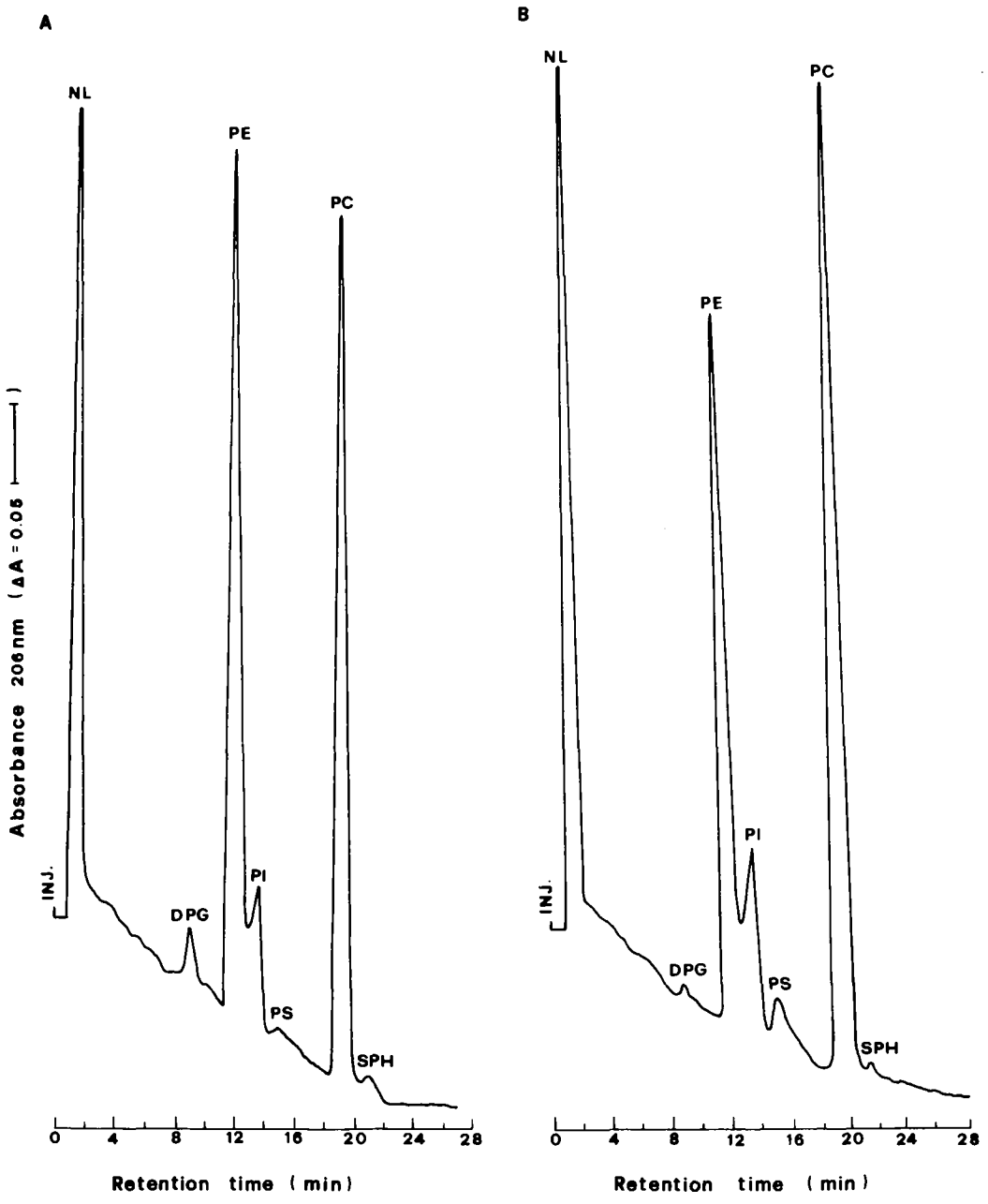

FIG. 1. HPLC separation of mitochondrial (A) and microsomal (B) phospholipids. NL = neutral lipids; DPG = cardiolipin; PE = phosphatidylethanolamine; PI = phosphatidylinositol; PS = phosphatidylserine; PC = phosphatidylcholine; SPH = sphingomyelin.

TABLE 3

Pattern of Fatty Acids of Total and Single Phospholipids in Mitochondria as Determined by HPLC

	Fatty acid distribution (mol %)[a]									
	Total phospholipid		Phosphatidylcholine		Phosphatidylethanolamine		Phosphatidylinositol		Cardiolipin	
Fatty acid	Eu.	Hyp.	Eu.	Hyp.	Eu.	Hyp.	Eu.	Hyp.	Eu.	Hyp.
16:0	18.1 ± 1.0	16.1 ± 0.9[f]	22.0 ± 1.8	22.2 ± 2.0	17.5 ± 2.0	14.3 ± 2.1	20.8 ± 1.6	24.5 ± 2.0[f]	11.5 ± 1.0	33.0 ± 1.5[c]
16:1	4.0 ± 0.5	6.2 ± 0.4[d]	4.5 ± 1.0	3.1 ± 0.4	7.3 ± 0.5	7.5 ± 1.0	4.1 ± 0.8	6.8 ± 1.0[e]	6.2 ± 0.8	7.4 ± 0.3
18:0	17.3 ± 1.2	16.0 ± 0.9	14.5 ± 0.7	16.2 ± 1.0[f]	17.2 ± 0.8	22.4 ± 1.0[d]	19.2 ± 1.0	22.4 ± 1.2[e]	7.3 ± 0.8	16.5 ± 1.3[c]
18:1	9.4 ± 0.8	10.2 ± 1.0	11.4 ± 0.6	10.7 ± 1.1	12.3 ± 0.7	7.1 ± 0.8[d]	15.3 ± 2.3	11.5 ± 2.0	16.9 ± 2.1	14.2 ± 1.6
18:2	15.6 ± 1.0	11.8 ± 0.8[d]	13.6 ± 0.5	9.7 ± 0.6[d]	6.7 ± 0.5	3.9 ± 0.3[d]	11.0 ± 0.8	6.2 ± 0.5[d]	52.5 ± 1.4	20.1 ± 1.3[c]
20:3	4.1 ± 0.5	1.6 ± 0.3[d]	2.0 ± 0.2	0.9 ± 0.3[d]	3.5 ± 0.09	1.5 ± 0.1[c]	3.1 ± 1.0	4.0 ± 0.9	2.4 ± 0.8	3.4 ± 1.3
20:4	28.2 ± 1.5	34.1 ± 2.0[e]	28.7 ± 1.3	33.2 ± 1.7[e]	31.2 ± 1.5	37.3 ± 1.0[d]	22.2 ± 2.0	21.5 ± 2.2	2.0 ± 0.3	3.0 ± 0.4[e]
22:6	3.3 ± 0.3	4.0 ± 0.5	3.2 ± 0.6	4.0 ± 0.4	4.3 ± 0.5	6.0 ± 0.3[d]	4.3 ± 0.9	3.1 ± 0.6	1.2 ± 0.2	2.4 ± 0.15[d]
UI[b]	189.5 ± 3.2	205.2 ± 4.1	183.1 ± 2.6	192.7 ± 3.1	194.1 ± 3.5	212.1 ± 2.7	165.3 ± 2.9	147.3 ± 2.5[d]	150.5 ± 5.1	98.4 ± 4.0[e]
20:4/18:2	1.81 ± 0.04	2.89 ± 0.05[c]	2.11 ± 0.06	3.42 ± 0.08[c]	4.66 ± 0.09	9.56 ± 0.11[c]	2.02 ± 0.23	3.47 ± 0.15[d]	0.04 ± 0.01	0.15 ± 0.03[d]

Eu = Euthyroid; Hyp = Hyperthyroid.

[a]Each value represents the mean obtained for 5 experiments with 8 rats each ± SE.

[b]The unsaturation index is defined as Σ mol % of each fatty acid X number of double bonds of the same fatty acid.

[c]$p < 0.001$ vs euthyroid.

[d]$p < 0.01$ vs euthyroid.

[e]$p < 0.02$ vs euthyroid.

[f]$p < 0.05$ vs euthyroid.

TABLE 4

Pattern of Fatty Acids of Total and Single Phospholipids in Microsomes as Determined by HPLC

	Fatty acid distribution (mol %)[a]							
	Total phospholipid		Phosphatidylcholine		Phosphatidylethanolamine		Phosphatidylinositol	
Fatty acid	Eu.	Hyp.	Eu.	Hyp.	Eu.	Hyp.	Eu.	Hyp.
16:0	19.6 ± 1.2	18.3 ± 1.9	22.7 ± 0.9	21.9 ± 1.6	18.5 ± 1.0	16.6 ± 1.4	20.7 ± 1.7	21.6 ± 1.3
16:1	5.2 ± 0.6	4.5 ± 0.4	3.3 ± 0.2	3.0 ± 0.3	6.2 ± 0.5	6.0 ± 0.7	6.0 ± 0.9	5.7 ± 0.6
18:0	16.3 ± 0.9	17.2 ± 1.0	13.6 ± 0.8	15.8 ± 0.6[d]	17.1 ± 0.9	20.2 ± 0.5[c]	21.8 ± 2.0	22.2 ± 1.5
18:1	14.4 ± 0.7	14.0 ± 1.1	15.1 ± 1.0	10.3 ± 0.7[c]	9.0 ± 0.6	8.1 ± 1.0	11.4 ± 0.9	11.5 ± 0.7
18:2	11.2 ± 0.8	10.1 ± 0.6	14.2 ± 0.9	16.0 ± 0.7	7.9 ± 0.6	6.8 ± 0.7	8.5 ± 0.8	8.9 ± 1.0
20:3	3.1 ± 0.3	1.8 ± 0.2[c]	2.7 ± 0.4	3.5 ± 0.5	4.9 ± 0.6	4.7 ± 0.3	2.0 ± 0.3	1.8 ± 0.4
20:4	28.1 ± 1.0	30.9 ± 1.3	25.9 ± 1.5	24.3 ± 1.1	30.6 ± 1.8	31.1 ± 2.0	26.4 ± 1.9	25.3 ± 1.5
22:6	2.1 ± 0.2	3.2 ± 0.3[c]	2.5 ± 0.5	5.2 ± 0.6[c]	5.8 ± 0.8	6.5 ± 0.4	3.2 ± 0.3	3.0 ± 0.5
UI[b]	176.3 ± 3.1	186.9 ± 4.0	173.5 ± 2.5	184.2 ± 3.2	202.9 ± 3.7	205.2 ± 3.0	165.2 ± 2.1	159.6 ± 3.2
20:4/18:2	2.51 ± 0.10	3.06 ± 0.09[c]	1.82 ± 0.12	1.52 ± 0.07[d]	3.87 ± 0.11	4.57 ± 0.09[f]	3.10 ± 0.15	2.84 ± 0.18

Eu = Euthyroid; Hyp = Hyperthyroid.

[a]Each value represents the mean obtained for 5 experiments with 8 rats each ± SE.

[b]The unsaturation index is defined as Σ mol % of each fatty acid X number of double bonds of the same fatty acid.

[c]$p < 0.01$ vs. euthyroid.

[d]$p < 0.02$ vs. euthyroid.

used as substrates (12).

Recently, Hulbert (11) has suggested that thyroid hormones act by altering the extent of membrane fatty acid unsaturation, hence membrane fluidity. Therefore, changes in membrane fluidity could be the manner in which the activities of many membrane-associated enzymes are altered by thyroid hormones (24-27).

The most frequent abnormalities reported in mitochondria from hypothyroid rats concern linoleic and arachidonic acid content alteration (23). In line with these findings, our results indicate that, in rat liver mitochondrial phospholipids from hyperthyroid rats, a decrease of 18:2 and an increase of 20:4 (Table 3) occurs. In addition, Table 3 shows that fatty acid composition of individual mitochondrial phospholipids can be altered in different ways by hyperthyroidism. Besides a linoleic acid decrease and a parallel arachidonic acid increase, a common characteristic of these compound is the higher percentage of stearic acid. The increase is probably caused by stimulated fatty acid chain elongation synthesis in this condition (12). Among phospholipids, cardiolipin exhibits a singular behavior. In this compound, a strong decrease in linoleic acid is accompanied by a palmitic and stearic acid increase in hyperthyroid rats. In these animals, this aspect could be caused by either a lower availability of linoleic acid owing to its stimulated conversion to arachidonic acid or to an higher oxidation (12, 28,29) with consequent substitution with palmitic and stearic acids. These acids are more available as their hepatic synthesis is almost doubled in hyperthyroid rats (12).

Since synthesis of unsaturated fatty acids occurs mainly in the microsomal fraction (30), we have analyzed the phospholipid fatty acid composition of these membranes from eu- and hyperthyroid rats. The data reported in Table 4 indicate that the fatty acid pattern of individual microsomal phospholipids is almost unaffected by hyperthyroidism. This data disagrees with the findings of Faas and Carter (25), thus confirming the possible existence of a polyunsaturated fatty acid pool accessible only to mitochondrial membranes, probably through the acylation-deacylation process whose metabolism is subjected to thyroid hormone regulation (31). Hoch et al. (26) and, more recently Faas and Carter (32) have reported a completely different situation in both liver mitochondria and microsomes of hypothyroid rats. In these subcellular compartments, a similar 20:4 decrease in favor of 18:2 was found. Moreover, these authors pointed out that hypothyroidism does not affect the acyl residues of cardiolipin at all.

The degree of fatty acyl unsaturation is not the only determinant of membrane fluidity. Cholesterol plays a key role because it appears to maintain the bilayer matrix in an intermediate fluid state by regulating the mobility of phospholipid fatty acyl chains (33). An increase in the amount of cholesterol in relation to phospholipid has been shown to decrease fluidity in both biological and artificial membranes (3,34). Hence, the strong decrease in total cholesterol/phospholipid molar ratio found in mitochondria from hyperthyroid rats (Table 1) could represent another important factor in the alteration of their membrane fluidity in this state (35).

The total cholesterol decrease found in the mitochondria and microsomes of hyperthyroid rats (Table 1) is probably caused by a high cholesterol turnover in these animals. A number of factors, e.g., the increased synthesis and oxidation of this compound in liver (15) and reduced gastrointestinal absorption, in association with increased excretion via the intestine (36), could contribute to this peculiarity. Recently, we reported that in hyperthyroid rats a major cholesterol transfer occurs from plasma to erythrocytes, suggesting that an additional factor, which is responsible for the plasma cholesterol decrease in these animals, can be caused by this phenomenon (37).

The synthesis of fatty acids (12,15,38) and phospholipids (17) in different rat tissues is stimulated by hyperthyroidism. Following this, in addition to that of plasma and red cell membranes already reported (37), an increase of liver phospholipid content has now been found in the present investigation.

Finally, the decrease in microsomal phospholipid content and the increase in mitochondrial phospholipids reported in Table 1 suggest that, in addition to other effects, through different phospholipid exchange proteins, thyroid hormones in liver could stimulate a net phospholipid transfer from microsomes to mitochondria.

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REFERENCES

1. Van deenen, L.L.M. (1965) in Progress in Chemistry of Fats and Other Lipids (Holman, R.T., ed.) Vol. 8, pp. 1-127, Pergamon Press, Oxford, England.
2. McMurchie, E.J., and Raison, J.K. (1979) Biochim. Biophys. Acta 554, 364-374.

3. Feo, F., Canuto, R.A., Garcea, R., and Gabriel, L. (1975) Biochim. Biophys. Acta 413, 116-134.
4. Borochov, H., Zahler, P., Wilbrandt, W., and Shinitzky, M. (1977) Biochim. Biophys. Acta 470, 382-388.
5. Shiga, T., Maeda, N., Suda, T., Konk, K., and Sekiya, M. (1979) Biochim. Biophys. Acta 553, 84-95.
6. Wolff, E.C., and Wolff, J. (1964) in The Thyroid Gland (Pitt-Rivers, R. and Trottner, W.R., eds.) Vol. 1 pp. 232-282, Butterworths, London, England.
7. Babior, B.M., Creagan, S., Ingbar, S.H., and Kipnes, R.S. (1973) Proc. Natl. Acad. Sci. USA 70, 98-102.
8. Ismail Beigi, F., and Edelman, I.S. (1970) Proc. Natl. Acad. Sci. USA 67, 1071-1078.
9. Gavaret, J.M., Vilkki, P., and Rall, J.E. (1979) FEBS Lett. 108, 40-44.
10. Hulbert, J., Augee, M.L., and Raison, J.K. (1976) Biochim. Biophys. Acta 455, 597-601.
11. Hulbert, J. (1978) J. Theor. Biol. 73, 81-100.
12. Landriscina, C., Gnoni, G.V., and Quagliariello, E. (1976) Eur. J. Biochem. 71, 135-143.
13. Booth, F.W., and Holloszy, J.O. (1975) Arch. Biochem. Biophys. 167, 674-677.
14. Gnoni, G.V., Landriscina, C., and Quagliariello, E. (1978) FEBS Lett. 94, 179-182.
15. Gnoni, G.V., Landriscina, C., and Quagliariello, E. (1980) Biochem. Med. 24, 336-347.
16. Roncari, D.A.K., and Murthy, V.K. (1975) J. Biol. Chem. 250, 4134-4138.
17. Young, D.L., and Lynen, F. (1969) J. Biol. Chem. 244, 377-383.
18. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
19. Duncan, I.W., Culbreth, P.H., and Burtis, C.A. (1979) J. Chromatogr. 162, 281-292.
20. Bligh, E.G., and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911-917.
21. Nakamura, G.R. (1952) Anal. Chem. 24, 1372.
22. Miller, R.A., Bussel, N.E., and Ricketts, C. (1978) J. Liquid Chromatogr. 3, 291-304.
23. Hoch, F.L., Subramanian, C., Dhopeshwarkar, G.A., and Mead, J.F. (1981) Lipids 16, 328-335.
24. Clejan, S., Collipp, P.J., and Maddaiah, V.T. (1980) Arch. Biochem. Biophys. 203, 744-752.
25. Faas, F.H., and Carter, W.J. (1981) Biochem. J. 193, 845-852.
26. Hoch, F.L., Depierre, J.W., and Ernster, L. (1980) Eur. J. Biochem. 109, 301-306.
27. Gnoni, G.V., Landriscina, C., Ruggiero, F.M., and Quagliariello, E. (1983) Biochim. Biophys. Acta 751, 271-279.
28. Landriscina, C., Megli, F.M., and Quagliariello, E. (1976) Lipids 11, 61-66.
29. Stakkedstad, J.A., and Bremer, J. (1982) Biochim. Biophys. Acta 711, 90-100.
30. Landriscina, C., Gnoni, G.V., and Quagliariello, E. (1972) Eur. J. Biochem. 29, 188-196.
31. Reitz, R.C., El-Sheikh, M., Lands, W.E.M., Ismail, I.A., and Gunstone, F.D. (1969) Biochim. Biophys. Acta 176, 480-490.
32. Faas, F.H., and Carter, W.J. (1982) Biochem. J. 207, 29-35.
33. Demel, R.A., and De Kruyff, B. (1976) Biochim. Biophys. Acta 457, 109-132.
34. Cooper, R.A., Leslie, M.H., Fischkoff, S., Shinitzky, M., and Sanford, J.S. (1978) Biochemistry 17, 327-331.
35. Lehninger, A.L. (1959) J. Biol. Chem. 234, 2187-2195.
36. Mathe, D., and Chevallier, F. (1976) Biochim. Biophys. Acta 441, 155-164.
37. Ruggiero, F.M., Landriscina, C., Gnoni, G.V., and Quagliariello, E. (1983) Horm. Metab. Res., in press.
38. Gnoni, G.V., Landriscina, C., and Quagliariello, E. (1980) FEBS Lett. 122, 37-40.

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Total Gangliosides, Ganglioside Species and the Activity of Neuraminidase in Different Brain Regions and Spinal Cord of Normal and Undernourished Rats

P. BHARGAVA,[a] P.S. RAO,[b] A. VAJRESHWARI[b] and R. SHANKAR,[*a] [a]*Department of Biochemistry, Institute of Medical Sciences, Banaras Hindu University, Varanasi-221005, India; and* [b]*National Institute of Nutrition, Tarnaka, Hyderabad 500007, India*

ABSTRACT

Total gangliosides, their concentrations and the distribution of individual ganglioside species were determined in the spinal cord, pons-medulla, midbrain, cerebellum, hypothalamus, cerebral cortex, olfactory lobes and the rest of the forebrain, of 18- and 33-day-old normal and food-restricted and rehabilitated rats. The activity of neuraminidase in the different regions of the brain and spinal cord was determined. Differences in total gangliosides as well as individual species in different regions of the brain were observed. Among brain regions, while total ganglioside content was significantly reduced in the case of the cerebellum, the hypothalamus was the most affected in the distribution of ganglioside species and the activity of neuraminidase was decreased. The significance of the distribution of ganglioside species in different brain regions in relation to the activity of neuraminidase is discussed.
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INTRODUCTION

The vulnerability of the developing brain to nutritional stress is fairly well documented (1-3). Alterations in brain lipids, especially those related to myelin, have received greater attention (4,5). However, information on the developmental profile of gangliosides under nutritional deprivation is scanty (6,7). The importance of gangliosides as neural components affecting cerebral activity was recognized in earlier studies by McIlwain (8,9). Some gangliosides have been implicated in the transport of biogenic amines (10). Studies carried out so far on the distribution of gangliosides are usually confined to whole brain (7,11). Because some regions of the brain alter in maturity in response to early undernutrition (12), the determination of the ganglioside profile of different regions of the brain and spinal cord was thought worthwhile. In addition, the relative activity of neuraminidase, which might influence their distribution, was also determined in these tissues.

The present investigation, therefore, proposes to determine the total and individual ganglioside species in the pons-medulla, midbrain, cerebellum, hypothalamus, olfactory lobes, cerebral cortex and the rest of the forebrain and in the spinal cord of normal and food-restricted and rehabilitated rats. The possible role of neuraminidase in affecting the concentration of individual ganglioside species is discussed.

*To whom correspondence should be addressed.

MATERIALS AND METHODS

Chemicals

All common chemicals used were of the highest purity available and preferably of analytical grade. Resorcinol, N-acetyl-neuraminic acid (NANA), colominic acid (sodium salt), 2-thiobarbituric acid and bovine serum albumin (BSA) were products of Sigma Chemical Co., St. Louis, MO. The ganglioside mixture from bovine brains, used as the standard on TLC plates, was also obtained from Sigma Chemical Co.

Experimental Design

Rats of the CFY/NIN strain were used as experimental animals. The pups were undernourished during the suckling period by restricting their feeding time. The method followed was that of Eayrs and Horn (13), modified by Culley and Mertz (14).

Half the pups from a normal-sized litter (10-12 pups in a litter) were kept away from the mother for 12 hr/day. The other half were left with the mother for 24 hr/day to serve as the control group. Undernutrition of this kind was continued from the 6th to the 17th day after birth. All pups were sacrificed on day 18.

For some experiments, pups were rehabilitated after 12 days of undernourishment by leaving them for 24 hr/day with the mother from the 18th day of age and subsequently weaning on the same diet.

Rats were sacrificed by decapitation. Intact whole brains were quickly taken out and chilled

on a petri dish over ice. The vertebral columns of the rats were opened and the whole spinal cord was also taken out. The different regions of the brain were dissected out following the method of McGeer et al. (15). For each region, tissues from 2-3 animals were pooled.

Determination of Gangliosides

The procedure followed for the determination of gangliosides was essentially that described by Harth et al. (16). Ganglioside NANA was estimated by the resorcinol:HCl reagent of Svennerholm (17) as modified by Miettinen and Luukkainen (18). A small volume of concentrated ganglioside extract containing ca. 10 μg NANA was spotted for separation of the ganglioside fractions by using thin layer chromatography. Silica Gel G coated plates of 250 mμ thickness were used. These were developed successively in 3 different solvent systems in different chambers that were previously saturated with the solvent mixtures as follows: (a) chloroform–plate was run to the top, which took 2 hr; (b) chloroform/methanol/water (70:30:4 v/v)–plate was run to $^4/_5$ of the height and (c) chloroform/methanol/2.5 N ammonia (60:35:8, v/v/v)–plates were run to $^2/_3$ of the height.

Ganglioside fractions (purplish-blue spots) were detected by spraying with resorcinol-HCl reagent (17) while phospholipids and other lipids appeared as brownish-yellow bands on the upper region of the plate.

The bands, after marking, were scraped from the plate and the ganglioside-NANA component was estimated using Svennerholm's resorcinol-HCl reagent (17) following the method of Suzuki (19).

Assay of Neuraminidase

A 10% aqueous homogenate of the different brain regions, obtained as described earlier, was prepared. The enzyme was assayed according to Uchida et al. (20) using Aminoff's (21) thiobarbituric acid reagent for estimating free NANA. Substrate and enzyme blanks were included in the assay. Protein was estimated after precipitation with trichloroacetic acid by the method of Lowry et al. (22).

RESULTS

Undernutrition imposed postnatally, by cutting down the feeding time to 12 hr a day, brought about a significant decrease both in body weight and brain weight of 18-day-old pups (Table 1). Although all the brain regions and the spinal cord, except pons-medulla, show a significant decrease in their mass, most affected were the cerebellum and spinal cord (unpublished data).

Total Gangliosides in Different Regions of Brain and Spinal Cord

The conventional procedure described by Suzuki (19) for extracting gangliosides was not feasible, because of the large number of samples and small tissue size. However, the method of estimating gangliosides described by Harth et al. (16), when adopted for whole brain as well as for different regions, gave values identical with those obtained by using Suzuki's (19) procedure. The data presented for total gangliosides were obtained by following the procedure of Harth et al. (16).

Total ganglioside concentration on a wet weight basis was lower in the spinal cord than

TABLE 1

Effect of Undernutrition and Subsequent Rehabilitation on Body Weight, Brain Weight and Ganglioside Content of Spinal Cord and Brain of Rat

Age (days)	Animals	Body weight (g)	Brain weight (mg)	Total ganglioside NANA (μg/g wet weight	
				Spinal cord	Whole brain
18	Control	34.0 ± 2.9	910.0 ± 45.0	383.8 ± 38.4 (8)	675.9 ± 46.2 (8)
	Undernourished (for 12 days)	16.1 ± 1.0[a]	710.8 ± 41.1[a]	430.2 ± 46.4[a] (8)	665.3 ± 64.8 (8)
33	Control	74.3 ± 2.7	962.1 ± 40.7	442.6 ± 25.0 (4)	714.2 ± 57.5 (4)
	Undernourished	41.3 ± 3.4[a]	894.1 ± 59.3[b]	441.4 ± 17.4 (4)	768.3 ± 62.6 (4)

The number of observations is given in parentheses. Values are mean ±SD. Level of significance was calculated by Students' *t* test.

[a] $P < 0.001$.

[b] $P < 0.05$.

in the whole brain. Undernutrition increased slightly in concentrations of total gangliosides in the case of the spinal cord (Table 1).

Regarding different regions of the brain, the cerebellum and pons-medulla show a relatively lower content of total gangliosides (less than 500 μg/g wet wt). Total ganglioside concentration did not seem to affect the regions of the brain as a result of undernutrition, although a slight decrease in the case of the midbrain and the cerebral cortex and an increase in the hypothalamus could be visualized in the undernourished rats (Table 2). Rehabilitation of undernourished rats for 15 days reversed these changes in ganglioside concentration (Table 2).

Although ganglioside concentration, if calculated as μg NANA/g wet wt, did not seem to change much from undernutrition, the total ganglioside content of the different CNS regions differed as shown in Figure 1. At 18 days of age, the total ganglioside content of the spinal cord and whole brain was found to be significantly lower. Among the brain regions, the cerebellum, cerebral cortex, olfactory lobes and residual forebrain showed a significant decrease. However, after rehabilitation, at 33 days of age, the total ganglioside content of the whole brain and the spinal cord returns to normal, although the cerebellum and residual forebrain still show a lower level (Fig. 1).

Distribution of Ganglioside Species in Different Brain Regions and Spinal Cord

The distribution of molecular species of gangliosides in different regions of the brain and spinal cord are given in Tables 3 and 4. The values shown in the table are the percentage of distribution of the total NANA among individual species of gangliosides.

Five major bands of ganglioside species were detected from the rat brains. The ganglioside profile for different regions was determined in 18-day-old controls and undernourished and rehabilitated groups of rats 33 days old.

The spinal cord of normal rats showed a greater proportion of GD_{1a} at 18 days, but GT_1 predominated at 33 days. GQ_1, which was barely detectable at 18 days, became evident at 33 days (Tables 3 and 4).

The spinal cords from undernourished rats showed a relative increase in GT_1; GD_{1a} and GM_1 showed a slight decrease in value. However, rehabilitation narrowed these differences in 15 days (Table 3).

At 18 days, GT_1 predominated in the pons-medulla, midbrain, cerebellum and hypothalamus of control rats, whereas GD_{1a} predominated in the cerebral cortex and the rest of the forebrain. In undernourished rats, GD_{1a} was

TABLE 2

Regional Distribution of Gangliosides in Brains of Control, Undernourished and Rehabilitated Rats[a]

(days)	Animal	Pons-medulla	Midbrain	Cerebellum	Hypothalamus	Residual forebrain	Cerebral cortex	Olfactory lobes
18	Control (8)	423.8 ± 156.9	738.9 ± 260.7	467.6 ± 64.2	679.4 ± 105.2	698.0 ± 89.0	747.2 ± 101.4	638. 6 ± 138.4
	Undernourished (for 12 days) (8)	474.0 ± 27.6	654.6 ± 219.0	440.8 ± 74.6	740.8 ± 191.6	724.6 ± 101.8	721.9 ± 104.8	557.6 ± 76.0
33	Control (4)	385.4 ± 95.0	521.6 ± 48.8	638.6 ± 63.0	778.4 ± 69.6	703.8 ± 33.1	837.0 ± 99.4	458.0 ± 63.6
	Undernourished (for 12 days) Rehabilitated (for 15 days) (4)	464.8 ± 26.7	672.4 ± 66.4	619.3 ± 49.8	742.0 ± 72.0	756.8 ± 62.7	896.4 ± 114.2	511.2 ± 50.9

Number of observations is given in parentheses. Values are mean ± SD.

[a]Total gangliosides (μg NANA/g wet wt).

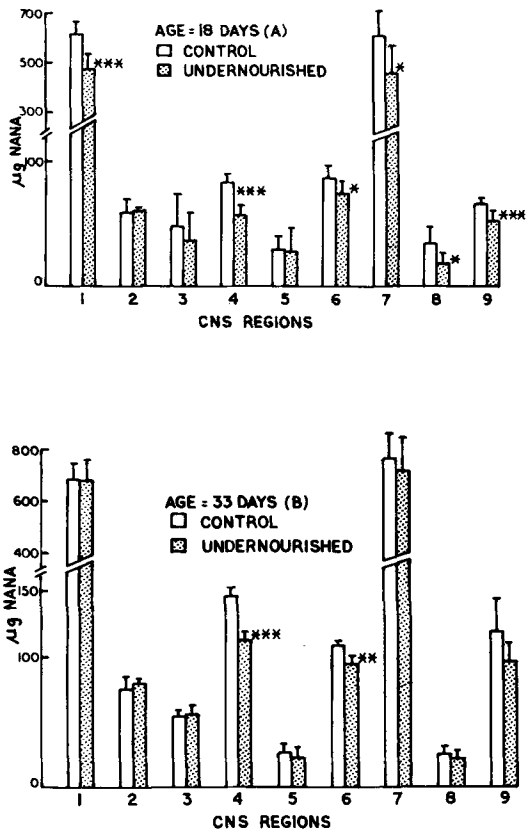

FIG. 1. Ganglioside content of whole brain, brain regions and spinal cord of normal, undernourished and rehabilitated rats. Rats were undernourished for 12 days (6th-17th day, postnatal age), A, and rehabilitated for 15 days (18th-32nd day, postnatal age), B. Ganglioside content is calculated in terms of μg of NANA obtained from whole tissue lipid extract. Number of observations for each value is 8 in A and 4 in B. Vertical lines show standard deviation.

1–whole brain, 2–pons-medulla, 3–midbrain, 4–cerebellum, 5–hypothalamus, 6–residual forebrain, 7–cerebral cortex, 8–olfactory lobes, and 9–spinal cord. *P < 0.02, **P < 0.01, ***P < 0.001.

higher in all the above regions and was lower in the hypothalamus at 18 days. A significantly higher proportion of GQ_1 was found in the hypothalamus. Of course, GQ_1 was hardly detectable in the different regions of the control rats (Table 3). Rehabilitation brought about a reversal in the response elicited as a result of undernutrition (Table 4).

Activity of Neuraminidase in Different Brain Regions and Spinal Cord

Colominic acid was used as substrate to assess neuraminidase activity. This was a perfect ganglioside mixture, in view of its ready solubility and as only one NANA residue is found that ensures suitable comparison. The relative activity of neuraminidase in the spinal cord and brain regions of control and undernourished rats are depicted in Table 5.

Significantly lower activity of the enzyme was evident in the case of the spinal cord at 18 days, which could not be restored by rehabilitation. The low activity may explain the higher level of GT_1 in the spinal cord of undernourished rats at 18 days.

Undernutrition did not significantly alter the activity of neuraminidase in the pons-medulla, cerebellum, cerebral cortex, olfactory lobes and rest of the forebrain, although a generalized lowering effect was evident from these regions of the brain.

A significant reduction in neuraminidase activity was evident in the hypothalamus, where a significantly high level of GQ_1, with a concomitant reduction in GD_{1a}, could be seen as a result of undernutrition. Undernutrition brought about a similar reduction in neuraminidase activity in the case of the midbrain, which coincides with increased levels of GT_1 and GQ_1 in this region.

DISCUSSION

Our results show that the total ganglioside content in the whole brain of control animals is not different from that of undernourished animals. These results agree with the observations made by Yusuf and Dickerson (23). However, other workers (24) showed a 14% reduction at 21 days of age.

Major alterations of total ganglioside content in the whole brain, spinal cord and cerebellum were caused by undernutrition. These differences persisted after rehabilitation, particularly in the case of the cerebellum. These observations indicate a reduced accretion of gangliosides in the feed-restricted pups.

The distribution of gangliosides in different brain regions of 33-day-old rats almost parallels the distribution of the aminergic pathways (25). Thus, the hypothalamus, through which most of the aminergic nerve bundles pass, showed a higher accretion of ganglioside NANA. The fact that gangliosides are concentrated more in gray matter is bound by the observation that brain regions like the cerebral cortex and the hypothalamus showed a higher concentration of ganglioside NANA than the pons-medulla and the midbrain, which are rich in white matter. This observation is in line with earlier observations of James and Fotherby (26). The procedure followed in this study for

TABLE 3

Effect of Undernutrition on the Distribution Pattern of Ganglioside Species in CNS of 18-Day-Old Rats

Region		GQ_1	GT_1	GD_{1b}	GD_{1a}	GM_1
Spinal cord						
Control	(4)	–	20.6 ± 5.1	27.7 ± 2.1	30.2 ± 6.7	21.5 ± 7.3
Undernourished	(4)	–	27.5 ± 10.2	28.9 ± 6.7	26.8 ± 0.6	16.8 ± 4.0
Pons-medulla						
Control	(7)	–	33.3 ± 8.4	21.9 ± 4.8	22.7 ± 3.4	22.1 ± 9.7
Undernourished	(7)	–	35.1 ± 6.2	22.9 ± 6.9	27.0 ± 2.5[b]	14.6 ± 2.2
Midbrain						
Control	(5)	–	36.4 ± 7.8	31.5 ± 5.0	20.5 ± 6.8	11.6 ± 3.7
Undernourished	(5)	9.9 ± 0.75	41.3 ± 3.5	19.9 ± 2.1[a]	20.4 ± 5.4	8.5 ± 2.4
Cerebellum						
Control	(6)	16.2 ± 3.3	31.7 ± 6.4	24.2 ± 3.3	16.3 ± 4.5	11.5 ± 3.6
Undernourished	(6)	17.9 ± 1.8	27.6 ± 3.0	14.9 ± 4.0[c]	21.7 ± 3.1	17.8 ± 4.8
Hypothalamus						
Control	(5)	7.4 ± 0.5	31.9 ± 3.2	22.6 ± 3.3	27.0 ± 3.2	11.4 ± 3.1
Undernourished	(5)	14.6 ± 1.7[d]	30.8 ± 1.1	20.3 ± 0.9	22.2 ± 1.6[a]	12.2 ± 2.4
Residual forebrain						
Control	(7)	–	35.8 ± 5.0	19.0 ± 7.5	27.5 ± 4.1	19.3 ± 6.5
Undernourished	(7)	–	34.8 ± 3.5	18.8 ± 6.8	34.1 ± 5.4[a]	12.3 ± 2.6[b]
Cerebral cortex						
Control	(10)	16.3 ± 3.3	24.6 ± 6.1	23.8 ± 5.3	22.2 ± 7.3	13.1 ± 3.0
Undernourished	(10)	15.8 ± 3.3	27.8 ± 9.9	21.6 ± 6.0	22.7 ± 9.1	12.0 ± 5.7
Olfactory lobes						
Control	(4)	–	30.1 ± 2.2	21.2 ± 6.8	36.4 ± 7.0	12.4 ± 2.8
Undernourished	(4)	–	22.2 ± 4.9[a]	26.1 ± 6.7	36.9 ± 3.4	14.7 ± 2.1

NANA was estimated in 5 major ganglioside species. Individual values are expressed as a percentage of the total NANA. All values are mean ± SD of the number of observations given in parentheses. A negative sign in a column shows the presence of a trace amount, which is not measurable.

[a] $P < 0.05$.

[b] $P < 0.02$.

[c] $P < 0.01$.

[d] $P < 0.001$.

the extraction of gangliosides was essentially that of Harth et al. (16). The method proved to be more convenient and avoids losses from the multiple steps involved in the original Folch et al. (27) and Suzuki (19) procedures, especially when a profile of ganglioside species is to be determined using lesser amounts of tissue.

Unlike bovine brain, which shows GT_1, GD_{1b}, GD_{1a} and GM_1 as major ganglioside species, rat brain showed, in addition, a fifth band that is confirmed as GQ_1 (28). GQ_1 becomes more prominent at later ages and is further enriched in phylogenetically older regions of the CNS, especially the spinal cord.

Observations made in this study show that higher gangliosides, like GT_1, were more concentrated in the cerebellum and pons-medulla, which mature earlier than the cerebral cortex and the rest of the forebrain, in which GD_{1a} predominates. This result is in line with the observations made by Merat and Dickerson (29), Ando et al. (28) and Harth et al. (16).

Although the total ganglioside concentration of different brain regions is least affected by undernutrition, the pattern of ganglioside species seems to be influenced as a result of nutritional stress. These effects are mostly on polysialo species. Higher levels of GQ_1 were found in the midbrain and the hypothalamus of undernourished rats at 18 days of age. In contrast, GD_{1b} was found to be significantly lower in the midbrain and cerebellum.

These alterations in ganglioside species as a result of undernutrition are significant from a functional viewpoint as gangliosides are associated with the maintenance of cerebral activity and cation transport (8,9) and can bind with certain biogenic amines that modulate brain function. For instance, histamine, which was recently recognized as an inhibitory neurotransmitter, binds with gangliosides and its binding depends on the number of sialic acid residues (30). Even the binding of serotonin, tryptamine and other drugs appears to depend on the NANA residues attached to gangliosides (31,32).

A possible relationship appears to exist between the increase of polysialogangliosides and neuraminidase activity in specific regions of the brain and spinal cord. Of particular impor-

TABLE 4

Effect of Undernutrition and Subsequent Rehabilitation on Pattern of Ganglioside Species in CNS of 33-Day-Old Rats (Percent Distribution of Total Ganglioside NANA)

Region		GQ_1	GT_1	GD_{1b}	GD_{1a}	GM_1
Spinal cord						
Control	(4)	14.6 ± 1.0	29.1 ± 5.9	21.9 ± 3.4	20.3 ± 1.8	13.3 ± 3.7
Undernourished	(4)	14.8 ± 2.6	29.4 ± 4.0	22.7 ± 0.7	18.4 ± 3.2	14.7 ± 3.2
Pons-medulla						
Control	(4)	10.7 ± 1.8	35.2 ± 3.1	28.0 ± 2.9	18.1 ± 4.0	9.7 ± 2.1
Undernourished	(4)	10.5 ± 2.0	33.8 ± 6.6	26.1 ± 1.6	19.1 ± 2.4	10.5 ± 2.0
Midbrain						
Control	(4)	15.2 ± 3.0	28.0 ± 6.6	23.3 ± 1.7	19.2 ± 0.9	14.3 ± 3.1
Undernourished	(4)	18.3 ± 1.9	29.1 ± 4.0	21.9 ± 1.4	17.2 ± 1.4	13.5 ± 2.0
Cerebellum						
Control	(4)	9.9 ± 0.5	38.3 ± 2.1	19.1 ± 2.8	27.1 ± 5.8	7.9 ± 1.2
Undernourished	(4)	9.8 ± 1.1	38.8 ± 4.2	17.2 ± 2.0	24.7 ± 3.5	9.4 ± 2.4
Hypothalamus						
Control	(4)	14.2 ± 2.0	25.5 ± 8.3	19.6 ± 2.6	24.6 ± 4.3	11.2 ± 1.3
Undernourished		12.1 ± 2.6	29.7 ± 1.8	20.3 ± 5.2	25.6 ± 1.3	12.4 ± 1.7
Residual forebrain						
Control	(4)	11.7 ± 1.4	25.9 ± 2.2	19.8 ± 0.9	27.0 ± 2.6	15.6 ± 1.1
Undernourished		12.0 ± 1.6	25.1 ± 3.5	19.6 ± 1.9	28.5 ± 2.8	14.8 ± 1.8
Cerebral cortex						
Control	(4)	11.8 ± 1.8	23.2 ± 1.9	17.0 ± 1.7	36.4 ± 3.7	14.3 ± 2.6
Undernourished		9.0 ± 0.6	25.3 ± 1.7	17.0 ± 1.9	35.1 ± 1.1	13.7 ± 1.3
Olfactory lobes						
Control	(4)	–	29.7 ± 2.3	20.2 ± 2.5	34.9 ± 2.6	15.1 ± 0.8
Undernourished	(4)	–	30.5 ± 2.2	20.2 ± 4.3	32.4 ± 6.3	16.9 ± 1.1

Rats were undernourished from 6th to 17th day by restricting their feeding time, then rehabilitated from 18th to 32nd day. Values are mean ± SD of the number of observations given in parentheses. A negative sign shows species was present but not in measurable quantity.

tance is the alteration in enzyme activity observed in the spinal cord, midbrain and hypothalamus. A higher content of GQ_1 in the hypothalamus and midbrain, with significantly decreased activity of neuraminidase, was evident in undernourished rats. These observations confirm similar findings from experiments on the whole brain of undernourished rats by Tyzbir and Dain (33) and Morgan and Naismith (34).

Researchers have argued that the use of artificial substrates for studying enzyme activity presents limitations in interpreting results, but we found in the present study that colominic acid served as a better substrate for neuraminidase than ganglioside mixtures. Moreover, a recent report shows that the reaction rate is proportional to the amount of enzyme only in the presence of water-soluble substrates and not with lipophilic ganglioside substrates (35).

The binding of biogenic amines to gangliosides is due to the negative charge of NANA residues. A fall in the total ganglioside content as well as the change in the proportion of the different ganglioside species, as seen in the present study, may result in a disturbance in the total charge distribution of gangliosides, which is expected to alter amine binding. In the present study, the hypothalamus was found to be maximally affected because of undernutrition. This region also contains many regulatory centers of the brain and amines are known to be involved in its various behavioral and emotional activities; therefore, an alteration in amine binding in this region caused by undernutrition may result in functional impairment in the brains of undernourished subjects.

Our studies point out the existence of differences in the distribution of ganglioside species in different brain regions whose concentration is under the influence of neuraminidase to a certain extent, which, in turn, could respond to nutritional deprivation.

TABLE 5

Effect of Undernutrition and Subsequent Rehabilitation on Neuraminidase Activity in Different Regions of Brain and Spinal Cord of Rat

Regions	18 Days of age (A)				33 Days of age (rehabilitated) (B)			
	Units/g wet weight		Specific activity		Units/g wet weight		Specific activity	
	C	U	C	U	C	U	C	U
Spinal cord	1557.0 ±404.0	895.0 ±222.0	18.0 ±4.4	11.4 ±3.7[a]	905.0 ±193.0	520.0 ±277.0	10.3 ±3.5	6.3 ±2.0
Pons-medulla	1307.0 ±248.0	1145.0 ±171.0	14.7 ±3.7	12.5 ±3.7	616.0 ±134.0	489.0 ±295.0	6.8 –	5.5 –
Midbrain	1446.0 ±337.0	1105.0 ±415.0	18.3 ±3.8	13.4 ±4.0[a]	893.0 ±342.0	1216.0 ±407.0	10.8 ±3.9	10.8 ±3.4
Cerebellum	1760.0 ±256.0	1813.0 ±475.0	19.0 ±3.8	18.0 ±3.9	1739.0 ±227.0	1031.0 ±174.0	15.9 ±0.5	14.5 ±1.3
Hypothalamus	1376.0 ±203.0	1000.0[b] ±294.0	16.8 ±2.9	10.7[c] ±3.4	977.0 ±268.0	905.0 ±419.0	12.1 ±2.1	12.2 ±5.4
Residual forebrain	1327.0 ±334.0	1092.0 ±231.0	15.6 ±3.7	13.1 ±3.4	1072.0 ±384.0	939.0 ±373.0	12.4 ±5.0	10.3 ±5.9
Cerebral cortex	1411.0 ±429.0	1046.0 ±76.0	13.3 ±3.6	12.0 ±3.0	1130.0 ±411.0	1126.0 ±306.0	9.4 ±0.9	10.9 ±4.3
Olfactory lobes	1145.0 ±354.0	1042.0 ±276.0	12.6 ±3.1	13.1 ±3.1	599.0 ±245.0	539.0 ±335.0	5.9 ±2.5	4.2 ±2.9

C = control. U = undernourished.

Rats were undernourished from 6th to 17th day (A) and rehabilitated from 18th to 32nd day (B). Enzyme was assayed in 10% aqueous homogenate using colominic acid as substrate by the method of Uchida et al. (20). One enzyme unit is taken as the amount required to liberate one nmole of free NANA/hr at 37 C. Specific activity = nmoles NANA/mg protein/hr. Number of observations ranged in A, 5-9 and in B, 3-6. Values are mean ± SD. Statistical significance was tested by Students' *t* test.

[a] $P < 0.05$.

[b] $P < 0.02$.

[c] $P < 0.01$.

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REFERENCES

1. Dobbing, J. (1972) in Lipids, Malnutrition and Developing Brain (Elliot, K., and Knighted, J., eds.) Ciba Foundation Symposium, pp. 9-20, Elsevier, Amsterdam, Netherlands.
2. Chase, H.P., Welch, N.N., Dabiere, S.S., Vasan, N.S., and Butterfield, L.J. (1972) Pediatrics 50, 403-411.
3. Winick, M. (1970) Fed. Proc. 29, 1510-1515.
4. Shankar, R., Katiyar, G.P., and Agrawal, K.N. (1975) Ind. J. Exp. Biol. 13, 563-564.
5. Chase, H.P., Dorsey, J., and McKhann, G.M. (1967) Pediatrics 40, 551-559.
6. Kokrady, S., Gopalakrishnamurthy, H.F., and Bachhawat, B.K. (1972) Proc. Nutr. Soc. Ind. 12, 20-31.
7. Vanier, M.T., Holm, M., Ohman, R., and Svennerholm, L. (1971) J. Neurochem. 18, 581-592.
8. Balakrishnan, S., and McIlwain, H. (1961) Biochem. J. 81, 72-78.
9. Thompson, C.G., and McIlwain, H. (1961) Biochem. J. 79, 342-347.
10. Burton, R.M., Howard, R.E., Baer, S., and Balfour, Y.M. (1964) Biochim. Biophys. Acta 84, 441-447.
11. Pritchard, E.T., and Cantin, P.L. (1962) Nature (Lond.) 193, 580-581.
12. Winick, M. (1975) Adv. Neurol. 13, 193-246.
13. Eayrs, J.T., and Horn, G. (1955) Anat. Rec. 121, 53-61.
14. Culley, W.J., and Mertz, E.T. (1965) Proc. Soc. Exp. Biol. Med. 118, 233-235.
15. McGeer, E.G., Gibson, S., Wada, J.A., and McGeer, P.L. (1967) Can. J. Biochem. 45, 1943-1952.
16. Harth, S., Dreyfus, H., Urban, P.F., and Mandel, P. (1978) Anal. Biochem. 86, 543-551.
17. Svennerholm, L. (1957) Biochim. Biophys. Acta 24, 604-611.
18. Miettinen, T., and Takki Luukkainen, L.T. (1959) Acta Chem. Scand. 13, 856-858.
19. Suzuki, K. (1964) Life Sci. 3, 1227-1233.
20. Uchida, Y., Trukada, Y., and Sugimori, T. (1977) J. Biochem. 82, 1425-1433.
21. Aminoff, D. (1959) Virology 7, 355-357.
22. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
23. Yusuf, H.K.M., and Dickerson, J.W.T. (1978) J. Biochem. 84, 1501-1506.
24. Reddy, P.V., and Sastry, P.S. (1978) Br. J. Nutr. 40, 403-411.

25. McGeer, P.L., Eccles, J.C., and McGeer, E.G. (1978) in Molecular Neurobiology of the Mammalian Brain, Plenum Press, NY.
26. James, F., and Fotherby, K. (1963) J. Neurochem. 10, 587-592.
27. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497-509.
28. Ando, S., Chang, N.C., and Yu, R.K. (1978) Anal. Biochem. 89, 437-450.
29. Merat, A., and Dickerson, J.W.T. (1973) J. Neurochem. 20, 873-880.
30. Alam, T., Cherian, R., and Balasubramanian, A.S. (1978) Ind. J. Biochem. Biophys. 15, 49-53.
31. Van Heyningen, W.E. (1963) J. Gen. Microbiol. 31, 375-387.
32. Ochoa, E.L.M., and Bangham, A.D. (1976) J. Neurochem. 26, 1193-1198.
33. Tyzbir, R.S., and Dain, J.A. (1972) Adv. Exp. Med. Biol. 19, 571-579.
34. Morgan, B.L.G., and Naismith, D.J. (1982) Br. J. Nutr. 48, 15-23.
35. Scheel, G., Acevedo, E., Conzelmann, E., Nehrkorn, H., and Sandhoff, K. (1982) Eur. J. Biochem. 127, 245-253.

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Long-chain Acyl-CoA Levels in Liver from Rats Fed High-Fat Diets: Is It of Significance for an Increased Peroxisomal β-Oxidation?

A. NILSSON, M.S. THOMASSEN*[1] and E. CHRISTIANSEN, *Institute for Nutrition Research, University of Oslo, Blindern, Oslo 3, Norway*

ABSTRACT

The levels of long-chain acyl-CoA in the livers of rats given diets containing various amounts of dietary oils were investigated. Increasing the amount of soybean oil in the diet from 5% to 25% (w/w) led to a 40% increase in long-chain acyl-CoA. With partially hydrogenated marine oil, a sigmoidal dose-response curve was obtained, giving a 60% increase when 20% or more of this oil was in the diet.

All high-fat diets tested resulted in higher levels of long-chain acyl-CoA than the low-fat control containing soybean oil. The increase was most prominent with partially hydrogenated marine and rapeseed oils.

With diets containing partially hydrogenated marine oil, the ratio of long-chain acyl-CoA to acid-soluble CoA was increased after 3 days, but decreased after 3 weeks, to a value similar to that observed in animals fed soybean oil because of an extensive increase in acid-soluble CoA.

Increased levels of long-chain acyl-CoA were also observed after clofibrate was administered, but the increase was less prominent than observed with high-fat diets.

When comparing the levels of long-chain acyl-CoA observed after 3 days on different diets with the peroxisomal β-oxidation activity previously determined after 3 weeks on the corresponding diets, a straight line was obtained. These results are discussed in relation to the possibility that long-chain acyl-CoA induces peroxisomal β-oxidation activity.

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INTRODUCTION

In 1976, Lazarow and de Duve disclosed the existence of a β-oxidation enzyme system in rat liver peroxisomes (1). Since then, numerous reports have appeared dealing with the effects of various drugs (2-4), and different metabolic conditions (5-8) on this enzyme system. However, at present, little is known about the subcellular mechanisms responsible for the observed alterations in peroxisomal biogenesis and enzyme activities. An increase in the cellular content of CoA has, however, been observed concomitantly with increased peroxisomal activities (9,10). Metabolic conditions with an increased peroxisomal β-oxidation activity, such as high-fat feeding (6,11), starvation (12) and alloxan-induced diabetes (7), also exhibit increased levels of long-chain acyl-CoA in the liver (13).

The possible role of long-chain acyl-CoA in the cellular events leading to increased peroxisomal β-oxidation has thus been suggested (14, 15). In previous studies, we have shown that high-fat diets may increase peroxisomal β-oxidation in both the liver and heart in rats, but, to a different extent, depending on the type of dietary oil (16,17). In the present investigation, we have examined the level of long-chain acyl-CoA in the liver resulting from feeding various dietary oils in an attempt to elucidate any possible correlation of this parameter to the previously described changes in peroxisomal β-oxidation activity.

*To whom correspondence should be addressed.
[1] Present address: Norwegian Food Research Institute, P.O. Box 50, N-1432 Aas-NLH, Norway.

EXPERIMENTAL

Materials

Dietary oils, including their analytical data, were obtained from DeNoFa and Lilleborg Fabriker A/S, Fredrikstad, Norway, except for the rapeseed oils, which were supplied by AB Karlshamns Oljefabriker, Karlshamn, Sweden. The fatty acid compositions are given in Table 1. Vitamin and salt mixtures were from ICN Pharmaceuticals, Cleveland, OH (Vit. diet fort. mix., Cat. no. 904654 and U.S.P. XVII, cat. no. 904610). Clofibrate (ethyl 2-(4-chlorophenoxy)-2-methyl-propionate) was obtained from Weiders Farmasøytiske A/S, Oslo, Norway. CoASH and thiodiglycol were obtained from the Sigma Chemical Co., St. Louis, MO. 2-Mercaptoethanol was purchased from BDH Chemicals Ltd., Poole, England. Palmitoyl-CoA was supplied by P-L Biochemicals Inc., Milwaukee, WI. Other chemicals were commercially available products of high purity.

Animals and Diets

Male weanling rats (60 g) of the Wistar strain

TABLE 1

Fatty Acid Composition as a Percentage of Dietary Oils[a]

Fatty acid	Soybean oil		Rapeseed oil		Marine oil	
	Unhydrogenated	Partially hydrogenated	Unhydrogenated	Partially hydrogenated	Unhydrogenated	Partially hydrogenated
14:0					6.7	6.8
16:0	10.9	10.6	4.9	4.1	14.7	12.8
16:1					9.5	9.9
18:0	3.8	14.0	1.0	6.3	1.0	3.0
18:1	24.3	73.8	13.5	32.5	16.1	15.1
18:2	52.5		20.0		1.6	2.5
18:3	7.5		7.9			
18:4					5.4	
20:0				1.7		2.4
20:1			6.2	6.5	12.8	16.4
20:2						4.3
20:5					9.0	
22:0				5.6		3.0
22:1			43.5	42.7	13.5	14.5
22:2						1.9
22:6					6.2	
Proportion of *trans* double bonds		0.55		0.65		0.55

[a]The fatty acid analyses were performed by the supplier. Only major fatty acids are included. The marine oils were obtained from capelin *(Mallotus villosus)*. The partially hydrogenated oils (except the rapeseed oil) were commercial, and not laboratory scale hydrogenated from the unhydrogenated oils listed in this table.

were purchased from Møllegaard Breeding Laboratory, Ejby, Denmark. The animals were fed a standard pellet diet for 5 days, then given the experimental, semisynthetic diets for 3 days or 3 weeks as described previously (16,18). The standard pellet diet was delivered from Møllesentralen A/S, Oslo, Norway. The main composition of this diet is meal from soybean, barley and wheat with 4% (w/w) fishmeal and 3% (w/w) milk powder, fortified with vitamins, amino acids and minerals. The protein and fat contents were 25% and 2% (w/w). The composition of the semisynthetic diets was, in weight percentage of the total: sucrose, 20.0%; corn starch, 52.8%, minus weight percentage of dietary oil; casein (with 2% methionine), 20%; cellulose 1.0%; vitamin mixture, 2.2%; salt mixture 4.0%; dietary oil, according to tables. The rats were housed in grid-bottomed cages, 2 in each cage, and had free access to food and water. The climatic conditions were: 23 C, 60% relative humidity, and a 12 hr light period (7 a.m.-7:00 p.m.). Some animals were treated with clofibrate for 3 or 10 days. The clofibrate was dissolved in acetone and mixed with the pellet diet (0.3%, w/w), and the acetone was then evaporated by air.

Preparation of Long-chain Acyl-CoA Precipitate from Rat Liver

The rats were killed at 10 a.m. They were stunned and decapitated and the abdominal cavity was quickly opened. The liver was lifted and clamped in situ between tongs cooled in liquid N_2. The liver samples were powdered in a stainless steel mortar cooled in liquid N_2 and stored at −70 C. Ca. 50 mg powdered liver was homogenized in 1.5 ml 7% (v/v) $HClO_4$ in centrifuge tubes. The samples were centrifuged at 18,000 rpm for 5 min in an Ole Dich microcentrifuge (Copenhagen, Denmark). The acid precipitates were washed with 1.5 ml cold 0.7% (v/v) $HClO_4$ and 1.5 ml distilled water. The washed precipitate was resuspended in 200 µl distilled water. 2-Mercaptoethanol (10 mM) was always present in the solutions.

Alkaline Hydrolysis of Acyl-CoA

The suspension or supernatant was adjusted to pH 11 with 1 N KOH and incubated in a water bath at 55 C for 60 min. After cooling in ice-water, the pH was adjusted to 5 with 7% (v/v) $HClO_4$ containing 0.5 M triethanolamine-HCl and centrifuged at 18,000 rpm (1,260 × g) for 5 min. Twenty to 40 µl of the resulting supernatant was injected directly into the liquid chromatograph for the assay of CoASH. The recovery was estimated by using a standard palmitoyl-CoA solution. The concentration of this standard solution was determined spectrophotometrically (19).

Assay of CoASH by High Pressure Liquid Chromatography (HPLC)

CoASH was measured by HPLC as described

by Ingebretsen et al. (19). A strong anion exchanger (Partisil-10 SAX, prepacked from Whatman in a 250 x 4.6 mm inner dimension stainless-steel tube), with a theoretical plate number per meter (N/m) of 19,500, was used. The mobile phase contained about 200 mM potassium-phosphate buffer, pH 3.9, 2% (v/v) isopropanol and 0.05% (v/v) thiodiglycol, which gave CoASH a retention time of ca. 8 min.

Protein Determination

The protein content of the powdered liver was determined as described by Lowry et al. (20), using bovine serum albumin as the standard.

Statistical Analysis

Student's *t*-test was used to evaluate the significance of differences between population means.

RESULTS

Long-chain Acyl-CoA Levels and Dietary Oils

That fat feeding is associated with elevated fatty acyl-CoA levels in the liver has been shown previously (13). No systematic study has, however, to our knowledge been conducted on the effects of different levels and different types of dietary oils on the cellular content of long-chain acyl-CoA.

In the present investigation, therefore, we fed rats diets containing different amounts of soybean oil as well as partially hydrogenated marine oil, and determined the resulting levels of long-chain acyl-CoA in the livers after 3 days. Table 2 shows the food consumption and weight gain of the rats given the different diets. With the partially hydrogenated marine oil diets, a slight decline was observed in both food consumption and weight gain in the groups given the diets containing the 2 highest levels of oil. With longer feeding periods (3 weeks), however, this effect is seen only in the animals receiving 30% (w/w) oil in the diet (16). The amounts of long-chain acyl-CoA found in the different dietary groups are presented in Figure 1. The results demonstrate that the increase in long-chain acyl-CoA levels from fat feeding is dependent on the type of dietary oil and the amount of oil in the diet. Thus, all partially hydrogenated marine oil diets tested resulted in higher levels than the ones containing soybean oil. This difference was especially prominent with 25% (w/w) or more oil in the diet. At this level, partially hydrogenated marine oil resulted in a level of long-chain acyl-CoA ca. 2 times as high as observed with the same amount of soybean oil, and ca. 3 times higher than that observed with the low-fat control containing soybean oil (5% SO) (Table 3). Furthermore, diets containing soybean oil seemed to give a linear relationship between the level of long-chain acyl-CoA and the amount of oil in the diet, whereas, with partially hydrogenated marine oil, a sigmoidal dose-dependency curve was observed. The most marked increase in long-chain acyl-CoA seem to occur with more than 20% (w/w) of this oil in the diet, which is also where the strongest effects on peroxisomal β-oxidation have been observed (16).

TABLE 2

Food Consumption and Weight Gains in Rats Fed Different Amounts and Kinds of Oils[a]

Dietary oil (%, w/w)		Food consumption (g/day)	Weight gain (g/day)
SO	5(5)	14.1 ± 0.5	6.0 ± 1.1
	15(5)	13.2 ± 1.4	5.8 ± 2.0
	25(5)	11.7 ± 0.2	6.4 ± 1.3
PHMO	5(5)	12.3 ± 1.0	7.5 ± 1.0
	10(6)	13.5 ± 1.5	7.3 ± 0.8
	15(4)	14.4 ± 1.3	6.5 ± 0.6
	20(5)	11.3 ± 1.5	6.3 ± 1.7
	25(6)	10.8 ± 1.2	5.3 ± 1.1
	30(4)	9.6 ± 0.3	4.1 ± 0.1
SO	20(6)	10.7 ± 1.0	4.8 ± 0.1
PHSO	20(6)	13.7 ± 4.1	4.6 ± 0.5
MO	20(5)	11.8 ± 0.8	4.5 ± 0.8
RO	20(5)	10.8 ± 0.6	3.9 ± 1.0
PHRO	20(6)	13.5 ± 0.7	4.2 ± 0.2

[a]Rats were fed the diets indicated for 3 days, with free access to food and water. The tabulated values represent means ± SD for the numbers of animals indicated in parentheses. Abbreviations used: SO, soybean oil; PHSO, partially hydrogenated soybean oil; MO, marine oil; PHMO, partially hydrogenated marine oil; RO, rapeseed oil; PHRO, partially hydrogenated rapeseed oil.

In a second experiment, we studied the effect of high-fat diets (20% w/w) containing various oils. The oils were of both plant and animal origin, and the effects of unhydrogenated oils were compared with those of the corresponding partially hydrogenated oils.

The results are presented in Table 3, along with the values obtained with a low-fat diet containing soybean oil. All high-fat diets revealed significantly higher levels of long-chain acyl-CoA than the low-fat control based on soybean oil. The increase was relatively small in high-fat diets containing unhydrogenated oils. The response from rapeseed oil was, however, slightly higher than with the 2 other oils tested. The partially hydrogenated rapeseed and

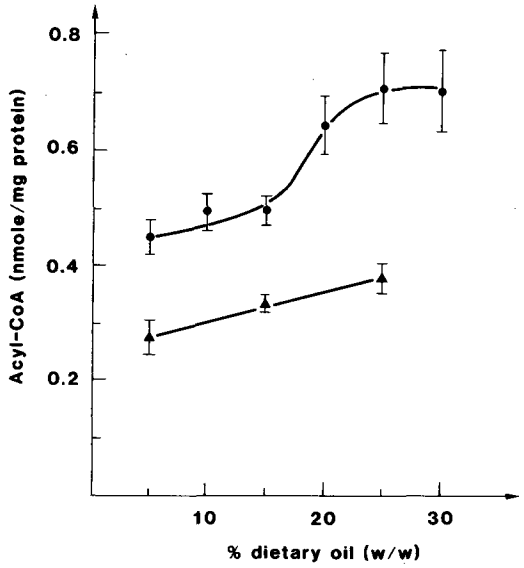

FIG. 1. Effect of various amounts of dietary oils on acyl-CoA levels in rat liver. Rats were fed on the diets indicated for 3 days, with free access to food and water. The long-chain acyl-CoA was determined as described in the Experimental section. The diets used were partially hydrogenated marine oil (•) and soybean oil (▲). The given values represent means ± SD (n = 5).

marine oils resulted in significantly higher levels of long-chain acyl-CoA than the corresponding unhydrogenated oils. This effect was not observed with the partially hydrogenated soybean oil.

TABLE 3

Effect of Different Oils on the Content of Acyl-CoA in Liver[a]

Dietary oil (%, w/w)		Long-chain acyl-CoA (nmol/mg protein)
SO	5(5)	0.27 ± 0.03
	20(6)	0.40 ± 0.02
PHSO	20(6)	0.41 ± 0.03
RO	20(5)	0.49 ± 0.07
PHRO	20(6)	0.70 ± 0.05[b]
MO	20(5)	0.43 ± 0.06
PHMO	20(5)	0.65 ± 0.05[b]

[a]Rats were fed the diets indicated for 3 days, with free access to food and water. The long-chain acyl-CoA was then determined as described in the Experimental section. The tabulated values represent means ± SD for the numbers of animals indicated in parentheses. The abbreviations are as in Table 1.

[b]Significantly different ($p < 0.01$) from the values obtained with the unhydrogenated oils.

Effect of Feeding Period and Clofibrate Administration

In previous studies (6,11,16), we demonstrated that feeding high-fat diets to rats for 3-4 weeks evokes adaptional changes in liver lipid metabolism. Therefore, studying any possible effects of the feeding period on the level of long-chain acyl-CoA in the liver was of interest. As can be seen from Table 4, no changes were observed because of the feeding period in the animals fed soybean oil or in the ones given partially hydrogenated marine oil.

High-fat feeding, and especially diets con-

TABLE 4

Effect of Feeding Period and Clofibrate Administration on Long-chain Acyl-CoA in Liver[a]

Dietary treatment		Feeding period	Long-chain acyl-CoA (nmol/mg protein)
SO	25 (5)	3 days	0.38 ± 0.02
SO	25 (5)	3 weeks	0.41 ± 0.03
PHMO	25 (6)	3 days	0.71 ± 0.06[+]
PHMO	25 (6)	3 weeks	0.70 ± 0.04[+]
Pellets	(5)	3 days	0.22 ± 0.02
Pellets	(6)	10 days	0.20 ± 0.03
Clofibrate	(6)	3 days	0.34 ± 0.07[++]
Clofibrate	(7)	10 days	0.27 ± 0.02[++]

[a]Rats were fed the diets indicated for different periods of time, and the content of long-chain acyl-CoA in the livers was determined as described in the Experimental section. The tabulated values are means ± SD for the numbers of animals indicated in parentheses. Abbreviations used: SO 25, 25% (w/w) soybean oil; PHMO 25, 25% (w/w) partially hydrogenated marine oil. The amount of clofibrate added to the pelleted diet was 0.3% (w/w). Statistically significant differences ($p < 0.01$) between the animals given SO and PHMO are indicated by +, and between the animals given pellets and ordinary pellets plus clofibrate by ++.

taining partially hydrogenated marine or rapeseed oils, results in changes in liver metabolism which, in some respects, are similar to the changes observed after the administration of the hypolipidemic drug clofibrate (16,21). The values observed with clofibrate added to the ordinary pelleted diet (0.3% w/w) for 3 days and 10 days, which are the most commonly used periods of treatment, are included in Table 4. A slight increase in the level of long-chain acyl-CoA was observed because of clofibrate feeding, the mean value being 1.4 times as high as in the control group (ordinary pelleted diet) after 10 days of treatment. Furthermore, the results seem to indicate that the level might be somewhat higher after 3 days of clofibrate administration (1.6 times that of the control). The difference between the values obtained after 3 and 10 days was, however, not statistically significant.

Changes in Acid-soluble CoA

Changes in the liver content of total CoA have been reported to occur from feeding partially hydrogenated marine oil (9) as well as during clofibrate administration (22). Therefore, the amount of acid-soluble CoA, including free CoASH and acetyl-CoA, was determined in some of the experiments described above. The results are presented in Table 5 along with the calculated ratios of long-chain acyl-CoA to acid-soluble CoA. After 3 days on the high-fat diets containing partially hydrogenated marine oil, no increases in acid-soluble CoA was detected when compared with the animals given either a high-fat diet based on soybean oil or an ordinary pelleted diet. Consequently, the ratio of long-chain acyl-CoA to acid-soluble CoA was increased in this group because of the increases described above in long-chain acyl-CoA levels.

After 3 weeks of feeding partially hydrogenated marine oil, however, a significant (ca. 100%) increase was observed in acid-soluble CoA. The constant level of long-chain acyl-CoA throughout the feeding period resulted in a decrease in the long-chain acyl-CoA to acid soluble CoA ratio from 3 days to 3 weeks on this diet.

Clofibrate administration, on the other hand, led to a significant increase in acid-soluble CoA even after 3 days, and a further increase was indicated after 10 days of administration. The level of acid-soluble CoA observed after 10 days of clofibrate administration was very similar to the level obtained when the rats were fed partially hydrogenated marine oil for 3 weeks. These time periods (10 days and 3 weeks) correspond to what is known to be required for optimal stimulation of peroxisomal β-oxidation activity by the 2 treatments (6,23).

TABLE 5

Effect of Dietary Treatment and Feeding Period on the Content of Acid-soluble CoA in Liver[a]

Dietary treatment		Feeding period	Acid-soluble CoA (nmol/mg protein)	Long-chain acyl-CoA/ acid-soluble CoA
SO	20(6)	3 days	1.09 ± 0.10	0.37 ± 0.05
PHMO	5(6)	3 days	0.94 ± 0.12	0.52 ± 0.08[b]
	10(5)	3 days	0.97 ± 0.17	0.52 ± 0.08[b]
	15(5)	3 days	1.01 ± 0.13	0.52 ± 0.07[b]
	20(6)	3 days	0.99 ± 0.14	0.63 ± 0.08[b]
	25(4)	3 days	0.93 ± 0.09	0.77 ± 0.09[b]
	30(4)	3 days	0.97 ± 0.09	0.73 ± 0.08[b]
PHMO	25(6)	3 weeks	1.84 ± 0.23[c]	0.39 ± 0.06[c]
Pellets	(6)	3 days	0.92 ± 0.08	0.22 ± 0.07
Clofibrate	(6)	3 days	1.54 ± 0.33[d]	0.23 ± 0.07
Clofibrate	(6)	10 days	1.77 ± 0.49[d]	0.17 ± 0.06

[a]Rats were fed the diets indicated for different periods of time, and acid-soluble CoA was determined as described in the Experimental section. The tabulated values represent means ± SD for the numbers of animals indicated in parentheses. The abbreviations are as in Tables 1 and 3.

[b]Significantly different ($p < 0.01$) from the values obtained with the animals given 20% (w/w) of SO.

[c]Significantly different ($p < 0.01$) from the values obtained after 3 days on the same diet.

[d]Significantly different ($p < 0.01$) from the values obtained with the animals given ordinary pellets.

The ratio of long-chain acyl-CoA to acid-soluble CoA was, at both periods of clofibrate administration, as low as, or lower than, the ratios observed with an ordinary pelleted diet.

DISCUSSION

Long-chain Acyl-CoA and High-fat Diets

Conditions associated with an excessive fatty acid supply to the liver, i.e., starvation and diabetes, have been found to result in an elevated intracellular content of long-chain acyl-CoA (13). High-fat feeding has also been found to increase long-chain acyl-CoA levels in the liver (13), an observation that is confirmed and extended by the present investigation. In addition, we provide evidence showing that large differences may occur in the content of tissue long-chain acyl-CoA derivatives, depending on the type of oil included in the diet.

The amount of long-chain acyl-CoA in the liver is likely to be determined by the balance between the rate of delivery of fatty acids to the liver, the rate of activation to acyl-CoA and their rate of use for esterification and oxidation. Of the dietary oils used in this study, the rapeseed and marine oils contained appreciable amounts of very long-chain monounsaturated fatty acids (20:1 and 22:1). Such fatty acids are believed to be more loosely bound to serum albumin than are shorter-chain fatty acids, e.g., stearic, oleic and linoleic acids (24). This loose binding might result in an increased influx of free fatty acids with diets containing such fatty acids. On the other hand, experiments with isolated hepatocytes have shown that erucic acid is, in fact, taken up and metabolized more slowly than palmitic acid (9).

The increased levels of long-chain acyl-CoA observed with dietary oils containing very long chain fatty acids may, instead, be the result of an inhibited metabolism of activated fatty acids in the liver. Thus, very long chain monoenoic acids, and especially 22:1-fatty acids, have been found to be metabolized more slowly than other, more ordinary fatty acids (e.g., palmitic and oleic acids) in a number of enzymatic reactions (14). Why the partially hydrogenated marine and rapeseed oils resulted in significantly higher levels of long-chain acyl-CoA than the corresponding unhydrogenated oils containing the same amounts of very long chain fatty acids is not readily evident. The hydrogenation process does, however, lead to the production of several geometrical and positional isomers of fatty acids (25,26), some of which may be unfavorable substrates in reactions using the corresponding long-chain acyl-CoA esters in the liver. In accordance with this explanation, an accumulation of long-chain acyl-CoA caused by a limited capacity for use, followed by product inhibition of the acyl-CoA synthetase (27) with 20% (w/w) or more oil in the diet may account for the sigmoidal character of the dose-dependency curve obtained with partially hydrogenated marine oil.

The results obtained with soybean oil and partially hydrogenated marine oil indicated that the difference in the level of long-chain acyl-CoA with different high-fat diets do persist on continued feeding. In fact, with partially hydrogenated marine oil, the same level of long-chain acyl-CoA was observed even after 3 months on the diet (results not shown). The increased ratio of long-chain acyl-CoA to acid-soluble CoA seen after 3 days, on the other hand, disappeared after 3 weeks of feeding from an increase in acid-soluble CoA in the animals given partially hydrogenated marine oil. A similar time dependency has been observed in the acyl-carnitine/carnitine ratio in rats fed rapeseed oil (28). Since an elevated acyl-carnitine/carnitine ratio as well as an elevated acyl-CoA/acid-soluble CoA ratio may reflect both increased and depressed fatty acid use, the significance of this finding is unclear. This drop in the ratios with time may, however, reflect the adaptive changes observed in the livers of rats given diets containing very long chain fatty acids, involving increased β-oxidation, especially in peroxisomes (9,11).

Long-chain Acyl-CoA and Peroxisomal β-Oxidation

We have previously demonstrated that feeding high-fat diets to rats leads to an increase in peroxisomal β-oxidation in the liver (16). In these studies, partially hydrogenated marine and rapeseed oil resulted in the most marked increase. Moreover, the dose-response curve of peroxisomal β-oxidation to different dietary concentrations of soybean oil and partially hydrogenated marine oil was very similar to the results presented for the level of long-chain acyl-CoA in the present study. Whereas the level of long-chain acyl-CoA seems to have reached its maximum after 3 days of feeding, the time-dependency of peroxisomal β-oxidation caused by feeding partially hydrogenated marine oil (6) indicated that this increase was a relatively slow adaptive response, reaching maximum level after ca. 3 weeks of feeding.

When the levels of long-chain acyl-CoA observed in the present study after 3 days were compared with the activity of peroxisomal β-oxidation previously observed after 3 weeks on the corresponding diets (16), a positive correlation was found (Fig. 2). These results may support the hypothesis that the cellular

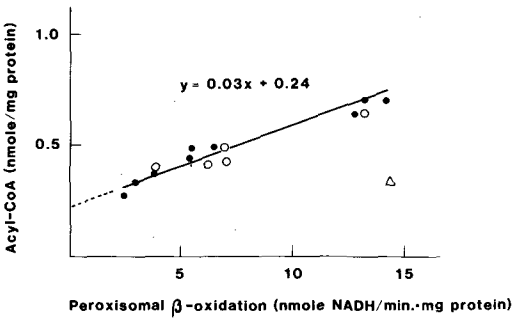

FIG. 2. Correlation between acyl-CoA levels and peroxisomal β-oxidation activity in rat liver. The acyl-CoA levels are taken from Figure 1, and Tables 2 and 3. The values of peroxisomal β-oxidation are taken from Thomassen et al. (16). Identical diets, (○); identical diets except for a small difference in percentage (w/w) of dietary oil (20% for acyl-CoA determination and 25% for peroxisomal β-oxidation), (●); clofibrate (0.3%) given for 10 days, (△); correlation coefficient r = 0.96.

level of long-chain acyl-CoA may act to induce peroxisomal β-oxidation. The possibility cannot be excluded, however, that both effects (increased long-chain acyl-CoA and peroxisomal β-oxidation) may be secondary to some other, at present unknown, factor.

The long-chain acyl-CoA levels observed after clofibrate feeding were significantly lower than the levels after feeding high doses of partially hydrogenated marine oil, in spite of the fact that the increase in peroxisomal β-oxidation by clofibrate is significantly higher than with partially hydrogenated marine oil (16). Also, the ratio of long-chain acyl-CoA to acid-soluble CoA is lower after clofibrate feeding. As observed by others (10,22), this ratio is, in fact, as low as or lower with clofibrate than with the control animals. This is caused by an extensive increase in acid-soluble CoA observed after 3 and 10 days of clofibrate administration. These observations may be in line with other findings that show that, even though both agents stimulate peroxisomal β-oxidation, the subcellular responses to feeding clofibrate and partially hydrogenated marine oil differ in many respects (21,29). Clofibrate is most likely activated to a CoA-ester (Normann and Flatmark, personal communication), as judged by a small increase in the AMP production when using the method for measuring acyl-CoA synthetase activity (30). To what extent this CoA derivative of clofibric acid is precipitated in the procedure used in this study is uncertain. The relatively low level of long-chain acyl-CoA observed in the animals fed clofibrate may, however, also suggest that not merely the acyl-CoA level, but also the acyl pattern may be of importance in determining the possible inducing effect.

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REFERENCES

1. Lazarow, P.B., and de Duve, C. (1976) Proc. Natl. Acad. Sci. USA 73, 2043-2046.
2. Lazarow, P.B. (1977) Science 197, 580-581.
3. Berge, R.K., and Bakke, O.M. (1981) Biochem. Pharmacol. 30, 2251-2256.
4. Osumi, T., and Hashimoto, T. (1978) Biochem. Biophys. Res. Commun. 83, 479-485.
5. Ishii, H., Fukumori, N., Horie, S., and Suga, T. (1980) Biochim. Biophys. Acta 617, 1-11.
6. Neat, C.E., Thomassen, M.S., and Osmundsen, H. (1980) Biochem. J. 186, 369-371.
7. Horie, S., Ishii, H., and Suga, T. (1981) J. Biochem. (Tokyo) 90, 1691-1696.
8. Reddy, J.K., Lalwani, N.D., Dabholkar, A.S., Reddy, M.K., and Qureshi, S.A. (1981) Biochem. Int. 3, 41-49.
9. Christiansen, R.Z., Christiansen, E.N., and Bremer, J. (1979) Biochim. Biophys. Acta 573, 417-429.
10. Berge, R.K., Aarsland, A., Bakke, O.M., and Farstad, M. (1983) Int. J. Biochem. 15, 191-204.
11. Neat, C.E., Thomassen, M.S., and Osmundsen, H. (1981) Biochem. J. 196, 149-159.
12. Ishii, H., Horie, S., and Suga, T. (1980) J. Biochem. (Tokyo) 87, 1855-1858.
13. Tubbs, P.K., and Garland, P.B. (1964) Biochem. J. 93, 550-557.
14. Bremer, J., and Norum, R.K. (1982) J. Lipid Res. 23, 243-256.
15. Christophersen, B.O., Norseth, J., Thomassen, M.S., Christiansen, E.N., Norum, K.R., Osmundsen, H., and Bremer, J. (1982) in Nutritional Evaluation of Long-chain Fatty Acids in Fish Oil (Barlow, S.M., and Stansby, M.E., eds.) pp. 89-139, Academic Press, London, England.
16. Thomassen, M.S., Christiansen, E.N., and Norum, K.R. (1982) Biochem. J. 206, 195-202.
17. Norseth, J., and Thomassen, M.S. (1983) Biochim. Biophys. Acta 751, 312-320.
18. Thomassen, M.S., Strøm, E., Christiansen, E.N., and Norum, K.R. (1979) Lipids 14, 58-65.
19. Ingebretsen, O.C., Normann, P.T., and Flatmark, T. (1979) Anal. Biochem. 96, 181-188.
20. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.S. (1951) J. Biol. Chem. 193, 265-275.
21. Christiansen, E.N., Flatmark, T., and Kryvi, H. (1981) Eur. J. Cell Biol. 26, 11-20.
22. Skrede, S., and Halvorsen, O. (1979) Eur. J. Biochem. 98, 223-229.
23. Krahling, J.B., and Tolbert, N.E. (1981) Arch. Biochem. Biophys. 209, 100-110.
24. Shafrir, E., Gatt, S., and Khasis, S. (1965) Biochim. Biophys. Acta 98, 365-371.
25. Hølmer, G., and Aaes-Jørgensen, E. (1969) Lipids 4, 507-514.

26. Conacher, H.B.S., Page, B.D., and Chadha, R.K. (1972) J. Am. Oil Chem. Soc. 49, 520-523.
27. Pande, S.V. (1973) Biochim. Biophys. Acta 306, 15-20.
28. Gumpen, S.A., and Norum, K.R. (1973) Biochim. Biophys. Acta 316, 48-55.
29. Flatmark, T., Christiansen, E.N., and Kryvi, H. (1981) Eur. J. cell Biol. 24, 62-69.
30. Normann, P.T., Thomassen, M.S., Christiansen, E.N., and Flatmark, T. (1981) Biochim. Biophys. Acta 664, 416-427.

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Influence of Acute Injection of Chloroquine on the Biliary Secretion of Lipids and Lysosomal Enzyme on Rats

H. LAFONT*[a], F. CHANUSSOT[a], C. DUPUY[a], P. LECHENE[b], D. LAIRON[a], M. CHARBONNIER-AUGEIRE[a], C. CHABERT[a], H. PORTUGAL[c], A.M. PAULI[c], and J.C. HAUTON[a], *[a]I.N.S.E.R.M. Unite 130, 70, Avenue Viton, 13009 Marseille, France; [b]I.N.S.E.R.M. Unite 31, 46, bd. de la Gaye, 13009 Marseille, France; and [c]Laboratoire central, Hopital Ste. Marguerite, 13009 Marseille, France*

ABSTRACT

Phospholipids and cholesterol combine with a protein fraction (IgA and an acid polypeptide) in bile to form the bile lipoprotein complex.

We wished to determine whether lysosomes participated only in IgA secretion or if their secretory role also involved the lipid components of the bile complex. This aspect was studied with a single acute injection of chloroquine, a lysosomotropic drug. The results show that a nonnegligible quantity of IgA travels through the lysosomes. In addition, phospholipid and cholesterol levels undergo a significant ($P<0.05$) decrease 1 hr after injection before increasing to normal levels. In contrast to the total inhibition of protein secretion (β-glucuronidase, acid phosphatase), a transitory decrease of the secretion of bile lipids takes place that suggest secretory mechanisms involving organelles other than lysosomes.
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INTRODUCTION

Bile phospholipids are tightly bound to a protein fraction, forming the bile lipoprotein complex (1,2). Bile salts participate by maintaining this complex in a more or less dispersed form. The protein fraction bound to the lipids, apo-bile lipoprotein complex (Apo BLC), is composed of 2 components. One shares antigenic properties with IgAs and the other is an anionic polypeptide fraction (2) detected in HDL (3).

Recent immunohistochemical results suggest that the intracellular pathway of the IgA fraction combined with the bile lipoprotein complex involves liver cell lysosomes (4). Thus, the possible participation of lysosomes in the combination of Apo BLC with bile lipids is a possibility to be verified.

La Russo et al. (5,6) formulated a hypothesis in which hepatic lysosomes participate in bile secretion. These authors suggested that this vesicular transport system could be the final common pathway of macromolecule secretion in the bile in liver cells. On the other hand, the same authors (7) demonstrated an inhibition of bile flow and of the secretion of bile lipids in the presence of ethynil estradiol, whereas the release of lysosomal enzymes increased in the bile. Previous results had also demonstrated that bile flow and secretion were dissociated from the secretion of lysosomal enzymes in cholostasis (5). These observations indicate that the lysosomes are not the only organelles involved in bile secretion.

In order to understand more fully the regulatory mechanisms of BLC secretion, we studied the eventual changes in the composition of the bile when the hepatic lysosomal system was modified by a lysosomotropic drug.

When chloroquine is administered chronically, it causes the accumulation of lipids in the liver (8,10,11), especially phospholipids (12,14), that result from the blockage of lysosomes. This blockage, in turn, results from the inhibition of lysosomal phospholipase A and C activities (12,15,16).

The present experiment involved the acute intoxication of rats in order to avoid any adaptation during drug detoxification that could interfere with the phenomenon we wished to study.

*To whom correspondence should be sent.

ABBREVIATIONS

IgA	: Immunoglobulin A
IgG	: Immunoglobulin G
CMP	: Cytidine monophosphate
BLC	: Bile lipoprotein complex
HDL	: High density lipoproteins
LDL	: Low density lipoproteins
VLDL	: Very low density lipoproteins

MATERIALS AND METHODS

Animals

Eleven male Wistar rats (IFFA-CREDO, L'Arbresle, France), 11 weeks old, were randomly placed in individual cages for a 2-week adaptation period in a temperature-controlled animal room. They were fed ad libitum with standard chow (U.A.R. No. A04, Villemoisson-sur-Orge, France).

On the day of the experiment, the 13-week-old rats weighed 358 ± 4 g.

Physiological Techniques and Samples

After being anesthesized with pentobarbital (5 mg/100 g body weight), the common bile duct was cannulated and bile recovered for 1 hr. At that time 6 rats received an intravenous (femoral vein) injection of 48.5 mg of chloroquine in 0.5 ml of 0.9% sodium chloride (treated rats). Total injection time was 5 min. The control group of 5 rats received 0.5 ml of saline in identical conditions. Bile was recovered every hour for 3 hr, immediately weighed and frozen.

The animals were then sacrificed by abdominal aorta puncture; the blood was collected over heparin. A piece of each liver was removed for histological and cytological study.

Assays

In the bile, phospholipids (17), bile salts (18), cholesterol (19) and the activities of two lysosomal enzymes, β-glucuronidase (20) and acid phosphatase (21) were assayed. IgA was assayed with the ACRA-ASSAY™ partigen kit (Miles-YEDA, England) using the radial diffusion method described by Mancini et al. (22).

In the plasma, triacylglycerols (23), total cholesterol (24), albumin (25), alkaline phosphatase (26), alamine amino transferase (27) and aspartate amino transferase (27) were assayed.

The results were compared statistically with Student's *t*-test or by a variance analysis.

Tissue Processing

Chemical fixation was with 1-2% glutaraldehyde or 2% paraformaldehyde/1% glutaraldehyde, each in 0.1 M cacodylate buffer (pH 7.2) for 2-6 hr at 4 C. After several washes in the same buffer, the specimens were dehydrated in a graded alcohol series and conventionally embedded in Epon 812.

Acid phosphatase cytochemistry. Small pieces of the liver fixed with either of the 2 methods explained above were incubated for 30 min at room temperature in Novikoff's medium for AcPase with CMP as substrate. Controls involved the same incubation, without CMP or in a complete medium in the presence of NaF, an AcPase inhibitor. The samples were then processed as above in alcohol and Epon. Semithin sections, 2-3 microns in thickness, were treated for 2-5 min with 2% aqueous ammonium sulfide to visualize reaction products.

RESULTS

Lysosomal Enzyme Activities in the Bile

The activities of 2 enzymes of lysosomal origin, β-glucuronidase (Fig. 1A) and acid phosphatase (Fig. 1B) remained stable in control rats during the 4 hr of the experiment. In the chloroquine-treated animals, however, a significant ($P<0.05$) inhibition of these activities occurred. Thus, 3 hr after injection, β-glucuronidase activity was one-third that of the controls and acid phosphatase activity was reduced by half.

Bile Secretion

The flow rate of bile was not affected by the drug and remained stable during the 4 hr of the experiment (Fig. 1C). The level of phospholipid secretion (Fig. 2A) in bile decreased with time, being relatively unaffected 1 hr after drug injection but decreasing significantly below that of the controls during the third hour of collection. The difference in cholesterol concentration between the control and chloroquine groups became significant after the second hour (Fig. 2B). The difference in bile-salt concentrations, on the other hand, was not significant (Fig. 2C).

The quantity of IgA remained constant in the bile of control rats (Fig. 2D), but decreased in the treated group, from 21.6 mg/dl to 4 mg/dl after 4 hr.

Plasmatic Biochemical Parameters

At the end of the experiment, the plasma levels of triacylglycerols, cholesterol and albumin were comparable in the treated and control groups (Table 1). Chloroquine tended to decrease plasma transaminase activity, while alkaline phosphatase levels remained unchanged.

Cytology and Histology

Liver samples were removed from control and treated rats, fixed and embedded. They were used to visualize lysosomes with the acid phosphatase reaction. The mean number of organelles per cell was determined and a variance analysis applied, enabling these numbers to be compared in the 2 series (Table 2). The number of lysosomes (counted in the cells after the cytochemical characterization of their contents) was increased significantly by chloroquine treatment ($F=0.01$).

After acute chloroquine treatment (Fig. 3), the major change noted in the liver cells was the abundance and the structure of lysosomes; they were more numerous at the periphery of bile canalicules and had a multilamellar structure. The images also suggested that autophagous processes were occurring frequently in the liver cell cytoplasm. These lysosomal images reacted positively to the acid phosphatase visualization reaction. They corresponded well to the reactive structures counted in the semithin sections on the basis of their pericanalicular localization.

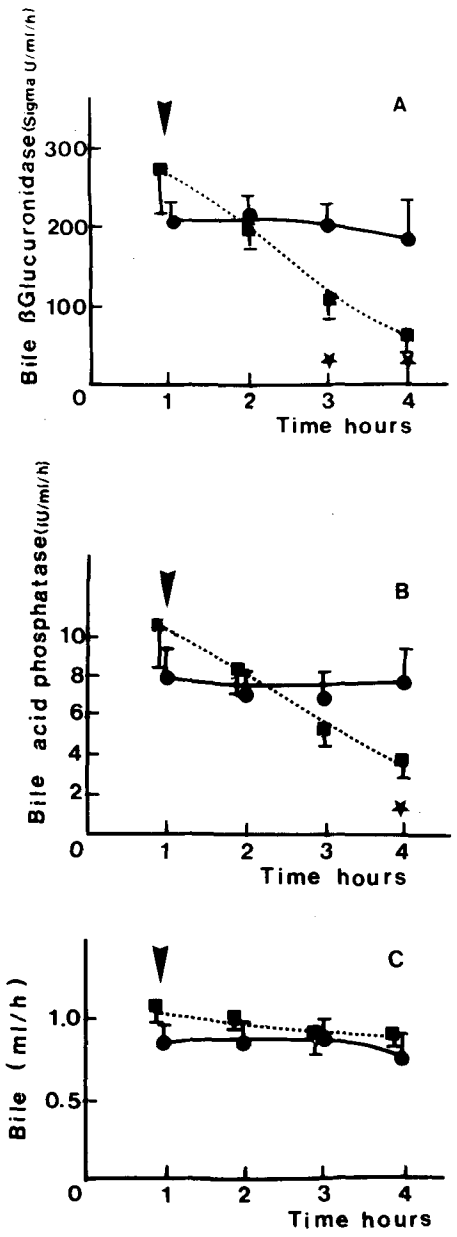

FIG. 1. The effect of chloroquine on the secretion of β-glucuronidase (A) and acid phosphatase (B) into bile and on bile flow (C). Results are expressed as means ± sem. n=5 for the control group o o n=6 for the chloroquine group □ □ Differences were analyzed with Student's *t*-test and are designated , when significantly different: $p<0.05$, $p<0.01$ (chloroquine-treated group vs control group). β-glucuronidase: Sigma unit/ml bile/hr = phenolphtalein concentration (μg/ml) × final volume (ml) of solution/bile volume (ml) assayed. Acid phosphatase: 1 IU = one micromole thymolphtalein produced per min under the specified conditions (20).

DISCUSSION

When administered chronically, chloroquine causes lipidosis in different tissues, especially hepetic tissues (9-11,13,15).

In the present series of acute intoxications, the drug decreased the activities of enzymes characteristic of hepatic lysosomal activity. β-Glucuronidase activity decreased by two-thirds and acid phosphatase activity was halved in relation to control values. As in chronic intoxication (14), lysosomal involvement was present. This was expressed by increased lysosomal enzyme activities in the liver as a result of the reduced secretion by the organelles. The effect of chloroquine on the secretion of bile lipids is more complex and transitory. The levels of phospholipids ($P<0.01$) and cholesterol ($P<0.05$) decreased significantly 2 or 3 hr after injection and then returned to their basal levels. Chloroquine had no effect on bile flow.

The ultrastructure of liver sections showed lipid storage forms, similar to the multilamellar bodies described by various authors (10-11). A significant increase occurred in the number of lysosomes localized around the bile canalicules in chloroquine-treated rats compared with controls.

Chloroquine is thus an inhibitor of lysosome secretion. The result is a decrease in the level of lysosomal enzymes secreted into the bile.

The present results, obtained in acute intoxication, can be compared with those described after chronic intoxication. In the chronic condition, a hepatic lipidosis develops as a result of the accumulation of cholesterol esters and phospholipids (10-11,13,15). This accumulation, in turn, results from the decreased metabolism of lysosomal phospholipids (8-9,13) and not from an increased rate of phospholipid transfer from their site of synthesis to the lysosomes (13).

Hostetler and Richman (28) stated that chloroquine-induced hepatic lipidosis would result from the free penetration of the drug into cells, the concentration of the drug in lysosomes, causing a direct block, or an indirect block resulting from a pH increase. The possibilities are not mutually exclusive.

The increase of the intralysosomal pH and the presence of the drug block phospholipase A and C activities (15) and cause the accumulation of phospholipids, primarily bis(monoacylglycero-)-phosphates. These compounds form a complex with chloroquine (29). This action is, nevertheless, reversible (15).

In the present case, there was no dose-effect relationship between the chloroquine blocking of lysosomes and the secretion of bile lipids. The decreased level of lipid secretion in the bile ($^1/_2$) is less than that of the decrease of lysosomal enzyme activities ($^2/_3$) in the bile, apparently indicating that

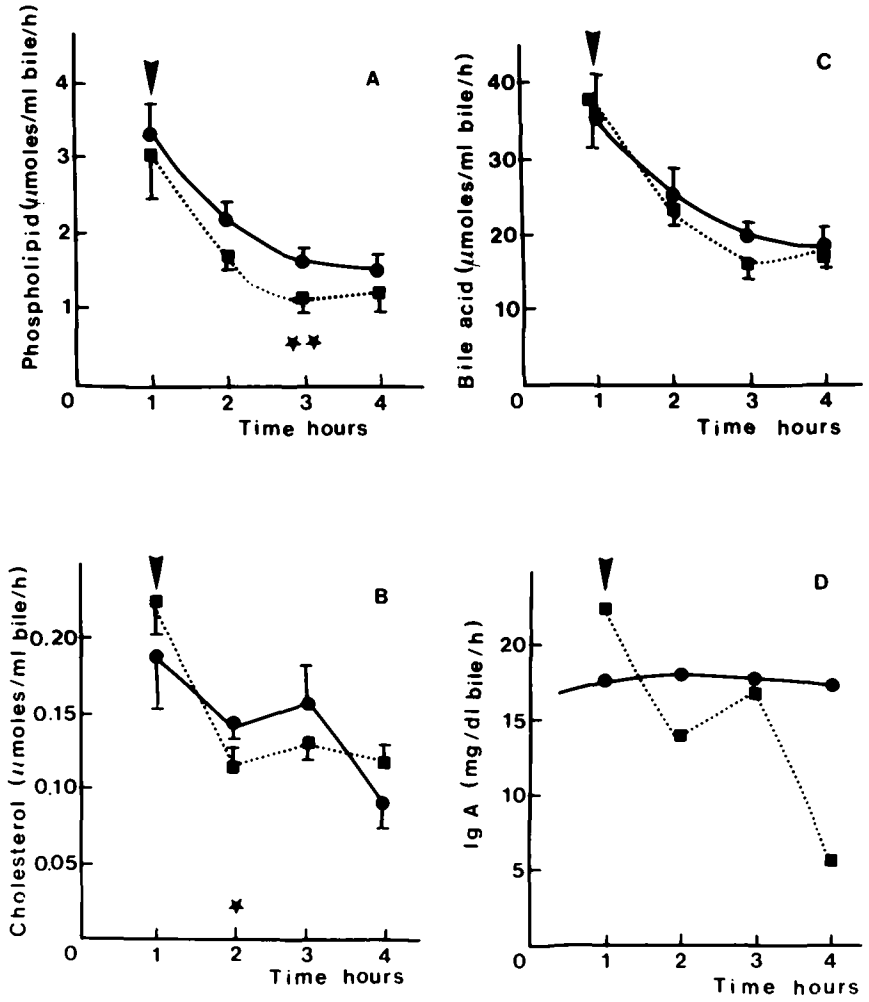

FIG. 2. The effect of chloroquine on the secretion of phospholipids (A), cholesterol (B), bile salts (C) and IgA (D) into bile. Results are expressed as means ± sem. n=5 for the control group ●——● n = 6 for the chloroquine group ■····■ Differences were analyzed with Student's *t*-test and are designated ☆ , ☆ ☆ when significantly different: ☆ $p<0.05$, ☆ ☆ $p<0.01$ (chloroquine-treated group vs control group).

the effect of the drug is not only at the level of lysosomal secretion.

In the case of acute chloroquine intoxication, the result would be that the secretion of bile lipids and the rate of bile flow would not be controlled only by mechanisms involving lysosomes. This conclusion is consistent with that of Lopez Del Pino and La Russo (7), who described this same type of dissociation in an experimental cholostasis induced by estrogens.

Immunological studies have shown the presence of serum proteins in the bile (30), e.g. albumin, IgA and IgG. The pathway of these proteins from the plasma to the bile is not clear. The involvement of the lysosomal system in the secretion of bile proteins is a hypothesis emphasized by de Duve (31). A dissociation between bile protein production and the secretion of lysosomal enzymes has been demonstrated in normal conditions (5). In the present work, however, the IgA fraction in the bile decreased considerably after chloroquine treatment, suggesting that a nonnegligible fraction of IgA destined for the bile traveled through the lysosome compartment or through organelles that could follow the same exocytosis mechanisms as lysosomes. Thus, the combination of the IgA fraction with bile lipids would occur only after their respective secretions in the bile duct and not in the liver cell.

Bile salts are indispensable for the secretion of phospholipids and cholesterol in the bile. Determining their influence on the secretion of lipids in

TABLE 1

Effect of Chloroquine on Plasma Biochemical Parameters

	Alkaline phosphatase IU/l	Aspartate aminotransferase IU/l	Alanine aminotransferase IU/l	Triacyl-glycerols mmole/l	Total cholesterol mmole/l	Albumin mole/l
Control rats	160±9	242±39	75±20	1.53±0.16	1.73±0.19	485±12
Chloroquine treated rats	150±18	172±21	62±6	1.53±0.15	1.73±0.16	495±8

Results are expressed as means ± sem. (n=4).

The blood was collected with heparin. After centrifugation, assays for alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, triacylglycerols, total cholesterol and albumin were performed by an automatic procedure with a SMAC autoanalyzer (Technicon Instruments Corp., Tarrytown, NY).

TABLE 2

Numbers of Lysosomes in Liver Cells of Controls and Chloroquine-treated Rats

Lysosomes/cell	Nontreated rat	Chloroquine-treated rat
Acid phosphatase-control with inhibitor of reaction	0.3±0.1	0.5±0.1
Acid phosphatase-complete reaction	7.6±0.8	11.2±1.2[F]

Variance analysis (differences are significant for F: 0.01).
Means ± sem.

the presence of chloroquine would make an interesting study. Bile salts antagonize the chloroquine block when perfused in vivo in animals previously intoxicated with an acute dose of chloroquine. In conditions comparable with those used in the present work, an additional perfusion of sodium taurocholate (20 micromoles/hr in the femoral vein to avoid lipid depletion of the bile over time) stimulated the secretion of bile lipids (unpublished results). Under these conditions, the activities of lysosomal enzymes in the bile of treated and control rats exhibited no great differences. The inhibitory effect of the drug on phospholipid secretion is masked by the antagonist effect of the perfused bile salts. This finding could be explained by the fact that the modalities of transfer of injected bile salts would be different from those of endogenous bile salts. The former cause a complex secretory effect of phospholipids (32) that would mask chloroquine inhibition.

The lysosomes are the sites of intense metabolic activity, including that of lipoproteins (33). Even so, acutely injected chloroquine does not affect the clearance of VLDL and LDL, although it inhibits the hepatic degradation of these lipoproteins (33). Our results of the assays of circulating triacylglycerols and cholesterol did not show a difference between the control and intoxicated groups. The concentration of plasma albumin also remained unchanged, since the protein synthesis capacity of the intoxicated liver was not affected. The stability or the nonsignificant decrease of alkaline phosphatase and hepatic transaminase levels in the presence of the drug could be the evidence of only moderate hepatic involvement by the chloroquine.

Work by Jones et al. (34) suggests that the Golgi apparatus plays a role in bile secretion in conjunction with the lysosomal system of the cell. Thus, a lysosome-to-bile hepatic pathway, as described by La Russo and Fowler (5), could be involved in the excretion of components from lipoproteins into the bile. The relation between lipoproteins and bile secretion was demonstrated by detecting Apo AI and Apo AII in the bile, the apoproteins arising from HDL.

The existence of these relations and the eventuality of regulatory mechanisms between lipoprotein metabolism and the secretion of lipids with plasma and bile destinies remain to be determined.

ACKNOWLEDGMENTS

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REFERENCES

1. Nalbone, G., Lafont, H., Lairon, D., Vigne, J.L., Domingo, N., Leonardi, J., and Hauton, J.C. (1978) Biochimie 60, 691-696.
2. Nalbone, G., Lafont, H., Vigne, J.L., Domingo, N., Lairon, D., Chabert, C., Lechene de la Porte, P., and Hauton, J.C. (1979) Biochemie 9, 1029-1041.
3. Vigne, J.L., Lafont, H., Nalbone, G., Domingo, N., Charbonnier-Augeire, M., Lairon, D., Leonardi, J., and Hauton, J.C. (1979) Biochem. Biophys. Res. Commun. 88, 1284-1287.
4. Lafont, H., Lechene de la Porte, P., Vigne, J.L., Nalbone, G., Lairon, D., Charbonnier-Augeire, C., and Hauton, J.C. (1982) Cytobios 34, 153-164.
5. La Russo, N.F., and Fowler, S. (1979) J. Clin. Invest. 64, 948-954.

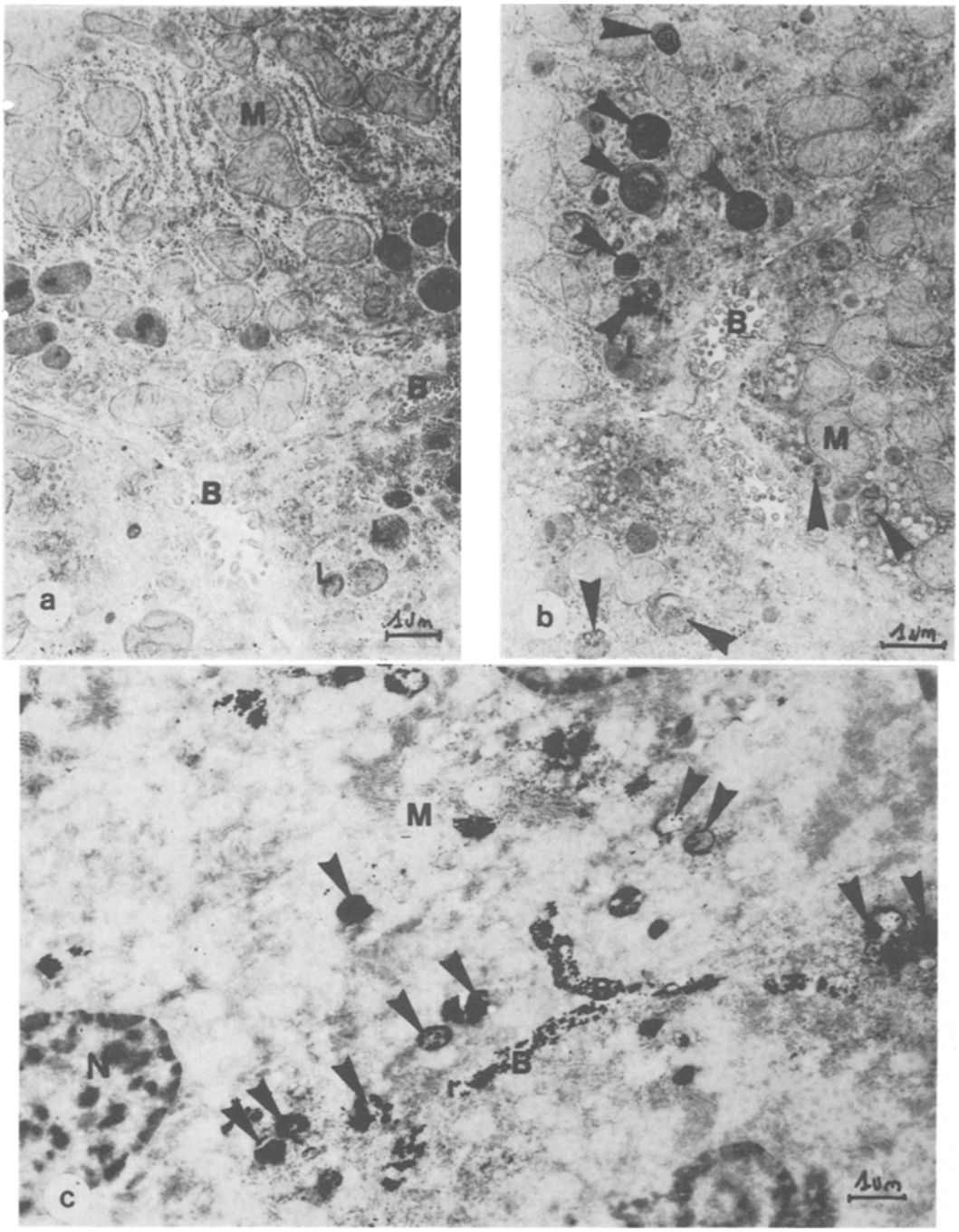

FIG. 3A. Control rat liver: the tissue was fixed with glutaraldehyde 1.5%. A post-fixation with osmium tetroxyde was performed. Lysosome (L) mitochondria (M) were normal, located near bile canaliculi (B) G × 10,000.

FIG. 3B. Rat liver after chloroquine treatment. Fixation with glutaraldehyde 1.5%, post-fixation with osmium tetroxyde. Lysosomes (L and arrow) were empty with multilamellar phase. Mitochondria (M) present a normal appearance. Bile canaliculi (B) G × 10,000.

FIG. 3C. Acid phosphatase. Activities localized after chloroquine treatment. Fixation with glutaraldehyde 1.5%. Numerous reactive lysosomes (L and arrow) were located near bile canaliculi (B) with mitochondria (M) G × 10,000.

6. La Russo, N.F., Kost, L.J., Carter, J.A., and Barham, S.S. (1982) Hepatology 2, 209-215.
7. Lopez Del Pino, V., and La Russo, N.F. (1981) J. Lipid Res. 22, 229-235.
8. Hostetler, K.Y., and Matsuzawa, Y. (1981) Biochem. Pharmacol. 30, 1121-1126.
9. Fredman, P., Klinghardt, G.W., Nilsson, O., and Svennerholm, L. (1982) Biochem. J. 201, 581-588.
10. Matsuzawa, Y., and Hostetler, K.Y. (1980) J. Lipid Res. 21, 202-214.
11. Tjiong, H.B., and Debuch, H. (1978) Hoppe Seyler's Z. Physiol. Chem. 359, 71-79.
12. Nachbaur, J., Colbeau, A., and Vignais, P.M. (1972) Biochim. Biophys. Acta 274, 426-446.
13. Matsuzawa, Y., and Hostetler, K.Y. (1980) Biochim. Biophys. Acta 620, 592-602.
14. Harder, A., and Debuch, H. (1982) Hoppe-Seyler's Z. Physiol. Chem. 363, 717-723.
15. Matsuzawa, Y., and Hostetler, K.Y. (1980) J. Biol. Chem. 255, 5190-5194.
16. Matsuzawa, Y., and Hostetler, K.Y. (1980) J. Biol. Chem. 255, 646-652.
17. Amic, J., Lairon, D., and Hauton, J.C. (1972) Clin. Chim. Acta 40, 107-114.
18. Domingo, N., Amic, J., and Hauton, J.C. (1972) Clin. Chim. Acta 37, 399-404.
19. Cholesterol; Colorimetric Method-Food Analysis, Boehringer-Mannheim.
20. β-Glucuronidase Colorimetric Procedure, Sigma Techn. Bulletin 325, 5-76.
21. Roy, A.V., Brower, M.E., and Hayden, J.E. (1971) Clin. Chim. 17, 1093.
22. Mancini, G., Carbonara, A.O., and Heremans, J.F. (1965) Immunochemistry 2, 235-254.
23. Bucolo, G., and David, H. (1973) Clin. Chem. 19, 475-483.
24. Schettler, G., and Nussel, E. (1975) Arbeitsmed. Sozialmed. Präventivmed. 10, 25-29.
25. Doumas, B.T., Watson, W.A., and Briggs, H.G. (1971) Clin. Chim. Acta 31, 87-96.
26. Morgensten, S., Kessler, G., and Auerbach, J. (1965) Clin. Chem. 11, 876-888.
27. Henry, J.R., Chiamori, M., and Golup Oj Berkman, S. (1960) Tech. Bull. Reg. Med. Tech. 30, 149-166.
28. Hostetler, K.Y., and Richman, D.D. (1982) Biochem. Pharmacol. 31, 3795-3799.
29. Harder, A., Pakalapati, G., and Debuch, H. (1981) Biochem. Biophys. Res. Commun. 99, 9-15.
30. Evans, W.H., Kremmer, T., and Culvenor, J.G. (1979) Biochem. J. 154, 589-595.
31. de Duve, C. (1974) Recherche 49, 815-826.
32. Gregory, D.H., Vlahcevic, Z.R., Merrill, F.P., and Swell, L. (1978) Gastroenterology 74, 93-100.
33. Stein, Y., Ebin, V., Bar-on, H., and Stein, O. (1977) Biochim. Biophys. Acta 486, 286-297.
34. Jones, A.L., Schmucker, D.L., Mooney, J.S., Adler, R.S., and Ockner, R.K. (1978) Anat. Rec. 192, 272-287.

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Diurnal Variation of Plasma Methyl Sterols and Cholesterol in the Rat: Relation to Hepatic Cholesterol Synthesis

T.E. STRANDBERG, R.S. TILVIS and T.A. MIETTINEN*, *Second Department of Medicine, University of Helsinki, 00290 Helsinki 29, Finland*

ABSTRACT

Free cholesterol of plasma low density lipoproteins (LDL) and high density lipoproteins (HDL) of the rat was high and that of plasma very low density lipoproteins (VLDL) was low during the dark period of the diurnal cycle. Variations in the esterified plasma sterols were inconsistent. Free methyl sterols were high in all lipoproteins during the dark phase. Simultaneously, the incorporation of ^{14}C-acetate into nonsaponifiable sterols and the concentrations of free methyl sterols and cholesterol in the liver were elevated.
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INTRODUCTION

Cholesterol synthesis undergoes circadian variations at least in rodents (1-4), swine (5) and chicken (6). The diurnal variation is most prominent in liver, but is also detectable in the mucosa of the small intestine. In ad libitum fed rats, the peak of synthesis activity is found in the middle of the dark period, at the time of active food intake.

The circadian rhythm of cholesterol synthesis has not been studied directly in man, but recent analyses of plasma cholesterol precursors, mevalonic acid (7) and squalene and methyl sterols (8) have revealed a nocturnal rise in their concentrations, suggesting activated cholesterol synthesis (9) at night. The results are quite unexpected, because the diurnal variation of cholesterol synthesis has been linked to the food intake at least in animal studies (10-12).

The present study was undertaken to elucidate the origin of plasma methyl sterols during the diurnal cycle. The results indicate a diurnal fluctuation of plasma and hepatic methyl sterol levels in the rat and suggest a relationship between this fluctuation and hepatic cholesterol synthesis.

MATERIALS AND METHODS

Male rats of the Sprague-Dawley strain (Anima Ltd, Finland) weighing 260-320 g were fed standard rat chow (Hankkija Ltd, Finland) and tap water ad libitum.

The animals were accustomed to alternating 12-hr periods of light and darkness for 2 weeks and were exsanguinated under ether anesthesia at different time points during a complete 24 hr diurnal cycle.

Plasma very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL) were separated from pooled samples of 3 rats by successive ultracentrifugation at densities 1.006 g/ml and 1.063 g/ml for 18 and 24 hr, respectively, in a Beckman Ti-50 rotor at 42,000 rev/min in a Sorvall-OTD-2 ultracentrifuge at 4 C. The bottom fraction of 1.063 g/ml was taken to represent the lipids in HDL (13).

Slices of liver were used for the in vitro studies of cholesterol synthesis. They were first preincubated for 10 min in 2 ml of Krebs-Ringer bicarbonate buffer containing 5.5 mmol glucose at 37 C, pH 7.4, and then the radioactive substrates, [1-^{14}C] acetate (5.95 Ci/mol) and DL-[2-^{3}H] mevalonic acid (2 Ci/mol) (Radiochemical Centre, Amersham, England) were added. The incubations were carried out under 95% O_2/5% CO_2 at 37 C, with shaking (100 oscillations/min) for 2 hr. The incubations were stopped by extracting the lipids in chloroform/methanol (2:1, v/v).

Lipids of total plasma, different lipoproteins and liver slices were extracted with chloroform/methanol (2:1, v/v), saponified, isolated with thin layer chromatography (TLC) and quantitated with gas liquid chromatography (GLC) (14). Qualitative analysis of different methyl sterol fractions (1-5) has been recently described (14). After extraction of the nonsaponifiable lipids (NSL) of the liver slices, the residue was acidified and free fatty acids were extracted. Aliquots were taken for the determination of radioactivities in a Wallac model 1215 Rackbeta liquid scintillation counter with 0.5% 2,5-diphenyloxazole (PPO) in toluene.

The measurement of cholesterol synthesis in vitro from ^{14}C-acetate and ^{3}H-mevalonate is described in detail earlier (15).

Statistical analysis was performed using the Student's t-test. A p value less than 0.05 was considered statistically significant.

RESULTS

Serial analysis of rat plasma revealed that plasma levels of free Δ8-methostenol, lanosterol and diunsaturated dimethylsterol and the sum of the 5 methyl sterols were low during the light period and high during the dark phase (Fig. 1). Since esterified methyl sterols showed inconsistent fluctuation, the percentage esterification of methyl sterols was

*To whom correspondence should be addressed.

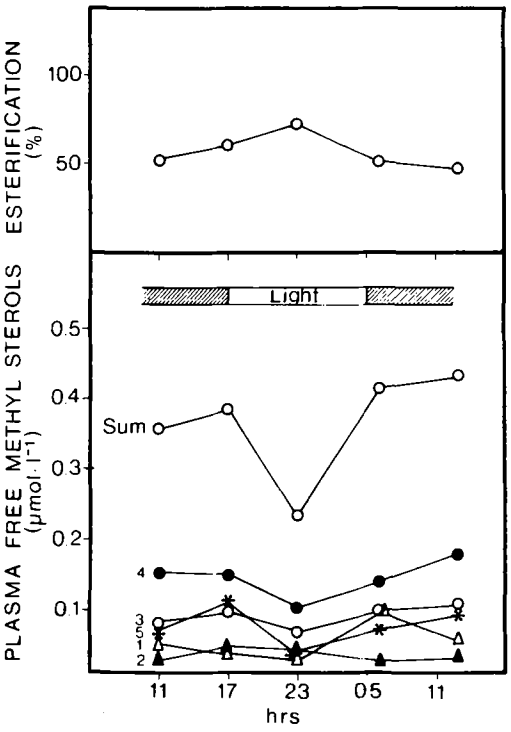

FIG. 1. Diurnal variation of plasma free methyl sterols (subfractions 1-5) and the esterification degree of plasma total methyl sterols. Each point is the mean of 2-5 rats. (△) Fraction 1 (8-en-monomethylsterol (4α-methyl-5α-cholest-8-en-3β-ol), also contains trace amounts of dihydrolanosterol (4,4,14α-trimethyl-5α-cholest-8-en-3β-ol). (△) Fraction 2 (monounsaturated dimethylsterol (4,4-dimethyl-5α-cholest-8-en-3β-ol). (o) Fraction 3 (methostenol (4α-methyl-5α-cholest-7-en-3β-ol). (o) Fraction 4 (lanosterol (4,4,14α-trimethyl-5α-cholest-8,24-dien-3β-ol). (*) Fraction 5 (diunsaturated dimethylsterol (4,4-dimethyl-5α-cholest-8(7), 24-dien-3β-ol).

clearly increased during the light period (Fig. 1).

To show which of the plasma lipoprotein fractions was responsible for the diurnal cycle, the methyl sterol and cholesterol levels were studied in plasma VLDL, LDL and HDL. Free VLDL-cholesterol was high during the light period, whereas free LDL and HDL cholesterol were significantly lower during the light than the dark phase (Table 1). Esterified cholesterol tended to vary in parallel to free cholesterol so that the percentage esterification of cholesterol remained unchanged in each lipoprotein.

In terms of pmol/μmol of total cholesterol, free methyl sterol contents of the 3 lipoproteins were more than twice as high during the dark than the light phase (Table 1). In similar terms, the esterified methyl sterol content was low during the dark period in LDL and unaffected by the lighting cycle in VLDL and HDL.

TABLE 1

Cholesterol and Methyl Sterol Levels of Plasma Lipoprotein in the Middle of the 12-hr Light and Dark Phases[a]

Phase	VLDL			LDL			HDL		
	free	ester	ester%	free	ester	ester%	free	ester	ester%
				Cholesterol (μmol/l)					
Light	73 ± 0.3	46 ± 8	38 ± 4	130 ± 13	360 ± 29	74 ± 1	97 ± 3	670 ± 93	87 ± 2
Dark	52 ± 8[b]	25 ± 4[b]	33 ± 5	190 ± 21[b]	460 ± 35	71 ± 1	130 ± 13[b]	760 ± 64	86 ± 1
				Methyl sterols[c] (pmol/μmol of cholesterol)					
Light	0.83 ± 0.02	2.30 ± 0.46	68 ± 1	0.13 ± 0.006	1.56 ± 0.14	92 ± 1	0.12 ± 0.02	0.20 ± 0.04	61 ± 6
Dark	1.93 ± 0.36[b]	3.04 ± 0.20	62 ± 5	0.30 ± 0.06[b]	60.98 ± 0.20[b]	76 ± 5[b]	0.26 ± 0.04[b]	0.26 ± 0.07	48 ± 3

[a]Values are mean ± SE (N=6).
[b]$p<0.05$, or less.
[c]Sum of individual methyl sterols.

To investigate the role of diurnal rhythm of hepatic cholesterol synthesis in the regulation of plasma methyl sterols, hepatic sterol concentration and the incorporation of ^{14}C-acetate into NSL (including squalene and sterols) were studied in the middle of the light and dark periods. The synthesis of NSL was 4 times higher in the dark period than in the light period, whereas the sterol synthesis from ^{3}H-mevalonate, as well as the incorporation of ^{14}C-acetate into fatty acids, was unaffected by the lighting periods throughout the day (Table 2). Consequently, the ^{14}C/^{3}H ratio of NSL was significantly augmented during the dark period.

The activated hepatic cholesterol synthesis was associated with increased hepatic concentrations of both free cholesterol and especially free methyl sterols, whereas the concentration of esterified sterols remained constant (Table 2).

DISCUSSION

In accordance with the earlier results (9), the high levels of plasma and hepatic free methyl sterols, and the simultaneous increase in hepatic cholesterol synthesis, demonstrate the role of hepatic cholesterol synthesis in the regulation of plasma methyl sterols in the rat. Furthermore, the present study indicates that the same regulation functions during the diurnal cycle. Despite relatively low plasma concentration of free methyl sterols and almost 4-fold fluctuation in hepatic cholesterol production, the diurnal rhythm of plasma methyl sterols is quite weak in the rat as compared to man (8). In humans, the diurnal fluctuation is up to 6-fold, in the rat ca. 2-fold. A reason may be that the hepatic pool of methyl sterols is fairly large in the rat, diluting the effect of altered methyl sterol production. Diurnal rhythm of plasma mevalonate is also low in the rat compared to that in man (7,16,17) and to variations in hepatic cholesterol synthesis (17).

Both in the rat and in man (8) plasma methyl sterols were high during the night and low during the day, irrespective of the fact that the rat eats during the dark period and man during the day. In man, the oscillation of plasma mevalonate disappears when food is omitted (7), suggesting that the diurnal rhythm of the plasma precursor level is associated with food intake and not with an intrinsic biological clock.

In the present study, the cholesterol content of VLDL exhibited a reciprocal variation to that of LDL and HDL. A diurnal variation with high daytime cholesterol levels in all lipoprotein classes is seen also in human subjects (18). Reciprocal eating patterns may explain this species difference in LDL and HDL, whereas the reason for the nocturnal decrease of VLDL cholesterol during high cholesterol synthesis in the rat is obscure.

It is likely that the diurnal variations of lipoprotein cholesterol levels are only partly determined by the fluctuation of endogenous cholesterol synthesis, as they are also affected by lipoprotein receptor activities, lipolytic enzymes, lecithin:cholesterol

TABLE 2

Hepatic Lipid Synthesis and Hepatic Sterol Concentrations in the Middle of the 12-hr Light and Dark Phases[a]

	Phase	
	Light	Dark
Incorporation of ^{14}C-acetate (nmol/g wet weight/2 hr)		
into NSL[b]	15±2	55±17[c]
into fatty acids	30±11	54±20
Incorporation of ^{3}H-mevalonate into NSL (nmol/g wet weight/2 hr)	625±84	679±136
Concentration of cholesterol (μmol/g wet weight)		
free	3.6±0.1	4.1±0.2[c]
ester	0.3±0.05	0.3±0.06
ester%	7.5±1.2	6.9±0.9
Concentration of methyl sterols (nmol/g wet weight)		
free	22.2±1.1	32.4±3.3[c]
ester	21.5±2.9	17.1±1.5
ester%	48.3±2.4	34.8±2.3[c]

[a]Values are mean ± SE (N = 6).
[b]Nonsaponifiable lipids, including squalene and sterols.
[c]$p<0.05$.

acyltransferase (LCAT) and cholesterol transfer between lipoprotein classes (cf. 19). Both lipoprotein lipase (20) and LCAT (21) show diurnal variation in man and the effects of these factors are diet- and possibly species-dependent.

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REFERENCES

1. Edwards, P.A., Muroya, H., and Gould, R.F. (1972) J. Lipid Res. 13, 396-401.
2. Shefer, S., Hauser, S., Lapar, V., and Mosbach, E.H. (1972) J. Lipid Res. 13, 571-573.
3. Ho, K.J. (1975) Proc. Soc. Exp. Biol. Med. 150, 271-277.
4. Mercer, N.J.H., and Holub, B.J. (1981) J. Lipid Res. 22, 792-799.
5. Rogers, D.H., Kim, D.N., Lee, K.T., Reiner, J.M., and Thomas, W.A. (1981) J. Lipid Res. 22, 822-819.
6. Ramirez, H., Alejaudre, M.J., and Garcia-Peregrin, E. (1982) Lipids 17, 434-436.
7. Parker, T.S., McNamara, D.J., Brown, C., Garrigan, O., Kolb, R., Batwin, H., and Ahrens, E.H., Jr. (1982) Proc. Natl. Acad. Sci. USA 79, 3037-3041.
8. Miettinen, T.A. (1982) J. Lipid Res. 23, 466-473.
9. Miettinen, T.A. (1969) Life Sci. 8, 713-721.
10. Dugan, R.E., Slakey, L.L., Breidis, A.V., and Porter, J.W. (1972) Arch. Biochem. Biophys. 152, 21-27.
11. Gregory, K.W., Smith, C.Z., and Booth, R. (1972) Biochem. J. 130, 1163-1165.
12. Ho, K.-J. (1979) Int. J. Chronobiol. 6, 39-50.
13. Havel, R.L., Eder, H.A., and Bragdon, J.H. (1955) J. Clin. Invest. 34, 1345-1353.
14. Tilvis, R., and Miettinen, T.A. (1980) Arch. Pathol. Lab. Med. 104, 35-40.
15. Strandberg, T.E., Tilvis, R.S., and Miettinen, T.A. (1981) Scand. J. Gastroent. 16, 801-810.
16. Popjak, G., Boehm, G., Parker, T.S., Edmond, J., Edwards, P.A., and Fogelman, A.M. (1979) J. Lipid Res. 20, 716-725.
17. Kopito, R.R., Weinstock, S.B., Freed, L.E., Murray, D.M., and Brunengraber, H. (1982) J. Lipid Res. 23, 577-583.
18. Miettinen, T.A. (1980) Ann. Clin. Res. 12, 295-298.
19. Myant, N.B. (1982) Clin. Sci. 62, 261-271.
20. Schlierf, G., Jessel, S., Ohm, J., Henck, C.C., Klose, G., Oster, P., Schellenberg, B., and Weizel, A. (1979) Eur. J. Clin. Invest. 9, 319-325.
21. Pagano Mirani-Oostdijk, C., Havekes, L., van der Voort, H., Jansson, H., and Terpsta, J. (1982) Eur. J. Clin. Invest. 12, 30.

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The Composition of Cardiac Phospholipids in Rats Fed Different Lipid Supplements

J.S. CHARNOCK,* M.Y. ABEYWARDENA, E.J. McMURCHIE and **G.R. RUSSELL,** *C.S.I.R.O., Division of Human Nutrition, Glenthorne Laboratory, Majors Road, O'Halloran Hill, South Australia 5158, Australia*

ABSTRACT

Changes in dietary lipid intake are known to alter the fatty acid composition of cardiac muscle of various animals. Because changes in cardiac muscle membrane structure and function may be involved in the pathogenesis of arrythmia and ischemia, we have examined the effects of dietary lipid supplements on the phospholipid distribution and fatty acid composition of rat atria and ventricle following 20 weeks feeding of diets supplemented with either 12% sunflower-seed oil or sheep fat.

Neither lipid supplement produced significant changes in the proportions of cholesterol, total phospholipids or phosphatidylcholine, phosphatidylethanolamine or diphosphatidylglycerol,—the phospholipid classes that together account for more than 90% of the total phospholipids of rat cardiac muscle. Significant changes were found in the profiles of the unsaturated fatty acids of all 3 phospholipid components of both atria and ventricle. Although similar, the changes between these tissues were not identical. However, in general, feeding a linoleic acid-rich sunflower seed oil supplement resulted in an increase in the ω-6 family of fatty acids, whereas feeding the relatively linoleic acid-poor sheep fat supplement decreased the level of ω-6 fatty acids but increased the levels of the ω-3 family, resulting in major shifts in the proportions of these families of acids. In particular, the ratio of arachidonic acid:docosahexaenoic acid (20:4, ω-6 / 22:6, ω-3), which is higher in all phospholipids of atria than ventricle, is increased by feeding linoleic acid, primarily by increasing the level of arachidonic acid in the muscle membranes. As dosahexaenoic acid does not occur in the diet, the increase in this acid which occurs after feeding animal fat, presumably arises from increased conversion of the small amounts of linolenic acid in all diets when the amount of linoleic acid present is reduced.

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INTRODUCTION

Manipulation of the dietary fat intake of several species of experimental animals has shown that the lipids of heart muscle are in a dynamic state that quite rapidly reflects alterations in the nature of the fat content of the diet (1-7). A recent study in our laboratory has demonstrated that differences in the mechanical performance (inotropic response to Ca^{2+} or isoprenaline) of isolated papillary muscles of the rat occur when the diet is supplemented with either sunflower-seed oil (SSO) or sheep kidney (perirenal) fat (SKF) (8). However, these dietary lipid supplements produced little or no effect on the inotropic response of isolated preparations of atrial muscle from these same animals. This observation might reflect the differential effects of dietary lipids on these anatomically and functionally distinct regions of the heart. Certainly, differences were apparent in the total phospholipid fatty acid composition of rat heart ventricle and atria that persisted after dietary lipid supplements (9).

The diet-induced differences in the mechanical performance of heart muscle may be related to changes in either the chemical or physical properties of cardiac muscle membranes, as changes in the lipid composition of a variety of membrane systems have been shown to modulate several integral membrane enzyme systems (10-12). Changes in the bioavailability of membrane phospholipid precursors for prostaglandin synthesis must also be considered since several studies have shown a strong relationship between dietary lipid intake, membrane phospholipid fatty acid composition and myocardial performance (13, 14). In a series of recent reviews, Katz and Messineo have drawn attention to the possible relationships between changes in cardiac membrane phospholipid composition, membrane function and the pathogenesis of cardiac ischemia and arrhythmia (15, 16). The extent of change in ratio of the classes of phospholipids or changes in the fatty acid composition of individual phospholipids were not determined in our earlier work (8,9).

This paper describes both the distribution of the major phospholipids, phosphatidylcholine (PC), phosphatidylethanolamine (PE) and diphosphatidylglycerol (cardiolipin, DPG), and their respective fatty acid compositions in rat ventricle and atria after 20 weeks of feeding dietary lipid supplements of widely different proportions of unsaturated:saturated fatty acids. The changes in the mechanical performance of the isolated heart muscle preparations that occur concurrently with this dietary treatment are to be described in detail elsewhere (Charnock J.S., McLennan P., Abeywardena M.Y. and Dryden W.F., manuscript in preparation).

MATERIALS AND METHODS

Thirty-six 80-day-old male Hooded Wistar rats

*To whom correspondence should be addressed.

weighing 295 ± 15 gm were assigned equally to 3 different dietary regimes to achieve widely different levels of fat intake or polyunsaturation:saturation (P/S) ratios. For the next 20 weeks the animals were fed either a commercially available rat chow (CC), containing 4% (^{W}/w) total fat, as the reference point for the study, or diets supplemented by the addition of 12% (^{W}/w) sunflower-seed oil (the SSO diet) obtained from Nuttelex Food Products Pty. Ltd., Victoria (Australia), or sheep kidney (perirenal) fat (the SKF diet) added to the commercial chow at the time of pelleting. Like the sunflower-seed oil used by De Deckere and Ten Hoor in their study of the effects of dietary fats on cardiac function (14), the sunflower seed oil used in these experiments was a rich source of linoleic acid (65% of the total fatty acids) and contained an appreciable amount of oleic acid (22%), but only small amounts of stearic acid (5%) and palmitic acid (6%).

Conversely, sheep kidney fat is a rich source of palmitic acid (24%) and stearic acid (41%), but contains only a small amount of linoleic acid (2%). However, its content of oleic acid (25%) is similar to that of sunflower-seed oil. Because of these wide differences in fatty acid composition, the P/S ratio of sunflower seed oil is > 5 whereas that of sheep kidney fat is < 0.1. Although not detectable in sunflower seed oil, about 0.1% (^{W}/w) of the total lipids of sheep kidney fat is cholesterol.

Elevating the total fat content of the diet, from 4% (^{W}/w) in the commercial rat chow to 16% (^{W}/w) in the diets with lipid supplements, increased the percentage of the total digestible energy of the diet that is obtainable from fats, i.e. the "energy percent" (en %), from about 12% in the reference diet (CC) to about 35% in both SSO and SKF supplemented diets. These latter values have been reported to be within the normal range for the Australian human population (17).

Combustion calorimetry of the commercial and refabricated diets demonstrated that both the lipid supplemented diets were essentially isocaloric (19.3 Kj/g and 18.6 Kj/g for the SSO and SKF diets, respectively). However, animals in these 2 groups consumed an average of only 20 gm of food per day, compared to 23 gm/day of the commercial diet (16.7 Kj/g) consumed by the control group.

After 20 weeks of feeding, the rats were sacrificed by decapitation under very light ether anesthesia. The hearts were removed and washed free of blood by immersion in oxygenated Krebs-Henseleit buffer (pH 7.4) before sections of the atrium and ventricle were collected for lipid analysis. Sections of tissue weighing 10-20 mg were dispersed in 2 ml of 0.25% (^{W}/w) aqueous butylated hydroxytoluene (BHT) by crushing in an all-glass Dounce homogenizer. After the addition of 4 ml of methyl alcohol and mixing by vortex, the samples were allowed to stand for 5 min at room temperature. Eight ml of chloroform were added and the tissue was homogenized by hand for several minutes until evenly dispersed. The mixture was centrifuged for 10 min at 800 *g* to separate the solvent layer. The aqueous phase was reextracted with 5 ml of chloroform and the pooled solvent fractions evaporated to dryness in a rotary vacuum apparatus before either estimation of the total phospholipid and cholesterol content or separation into the various phospholipid classes.

The dried lipid extract was taken up in 0.2 ml $CHCl_3$ and 50 µl aliquots applied to Silica Gel H plates for thin layer chromatography (TLC) and run in a first solvent system of 1:3 acetone/petroleum ether (40-60C). After development, the plates were dried in vacuo and rerun in the same direction in a second solvent of chloroform/methanol/water (65: 25:4, v/v/v) for separation of the major classes of phospholipid according to the method of Skipski and Barclay (18). Phospholipids were visualized under ultraviolet light after spraying the plates with 2'7'-dichlorofluorescein.

Samples were run in duplicate, one being for lipid analysis by gas liquid chromatography (GLC) by the method described previously (9), and the other for phosphate analysis by the method of Bowyer and King (19). The cholesterol content of the lipid extracts was also estimated by a GLC procedure described by Macgee et al. (20). Protein was determined by the method of Peterson (21). The percentage fatty acid composition of cardiac membrane phospholipids was expressed as the mean ± SEM and was compared for differences between ventricle and atrium or between diets, using an analysis of variance with Scheffe's procedure for multiple comparisons between pairs (22).

RESULTS

When added to the commercial laboratory chow (CC), the lipid supplements provided diets with widely different levels of P/S ratios, as shown in Table 1.

As expected, supplementing the commercial diet with SSO results in an increase in the proportion of linoleic acid (18:2 ω-6), and a decrease in the proportion of palmitic (16:0) and stearic acids (18:0) in the diet, whereas the diet supplemented with SKF contains increased proportions of palmitic (16:0) and stearic (18:0) acids as well as oleic acid (18:1 ω-9), which represents 25% of the total fatty acids present in sheep kidney (perirenal) fat. Supplementing the commercial diet with SKF also results in a significant decrease in the linoleic acid content in comparison to either the reference diet (CC) or that supplemented with SSO. However, the amount of this essential fatty acid (EFA) is sufficient to sustain healthy normal growth in these

TABLE 1

Composition of Fabricated Rat Diets

Fatty Acid (C:d, ω-x)	CC n = 14	SSO n = 23	SKF n = 24
14:0	1.6±0.1	0.3±0.1	2.6±0.1
16:0	20.8±0.1	9.2±0.2	22.3±0.3
16:1	3.2±0.2	0.6±0.1	1.6±0.1
17:0	0.9±0.4	—	1.5±0.1
18:0	7.2±0.4	5.6±0.1	29.4±0.8
18:1	22.2±0.2	22.8±0.2	29.8±0.7
18:2 ω-6	32.9±0.8	58.0±0.5	7.1±0.3
18:3 ω-3	2.6±0.1	0.8±0.1	1.1±0.1
20:0	0.1±0.1	0.3±0.1	0.6±0.1
20:1	3.1±0.3	0.7±0.1	0.6±0.1
22:0	—	0.7±0.1	—
22:1	3.6±0.3	0.7±0.1	0.5±0.1
24:0	—	0.2±0.1	—
22:6 ω-3	1.6±0.2	0.3±0.1	0.3±0.1
% Σ SAT	30.6±1.6	16.3±0.5	56.4±1.3
% Σ UNSAT	69.2±2.1	83.7±1.5	41.0±1.4
P/S	1.2	3.6	0.2
Cholesterol (%/w%)	.025	.023	.036

CC = Commercial rat chow; SSO = sunflower seed oil; SKF = sheep kidney fat.

Values shown are % total fatty acid (mean ± SEM, n = number of samples); — represents detectable amounts of fatty acids less than 0.1%. Small amounts of other minor fatty acid components of uncertain identity have been omitted from the table. The nomenclature for fatty acid identification (C:d, ω-x) refers to the chain length of the fatty acid (C), the number of double bonds (:d) and the number of carbon atoms (ω-x) from the last double bond to the methyl end of the molecule. P/S = Σ polyunsaturated fatty acids:Σ saturated fatty acids, but excludes the monoenes 16:1, 18:1, and 20:1. The preparation of the diets CC, SSO and SKF is described under Materials and Methods.

animals and is above the minimum level reported to be necessary to avoid EFA deficiency in male rats (23).

The percentage cholesterol content (%/w) of all 3 diets is low; that of the CC and SSO diets is ca. .025% and the diet supplemented with SKF is higher (.036%). Feeding the SKF diet for 20 weeks resulted in a small but significant ($p<.05$) increase in plasma cholesterol concentration (96 mg/100 ml) over that found after feeding either the SSO or the CC diet (89 and 85 mg/100 ml, respectively). All animals (12 per dietary group) gained weight throughout the experimental period, the rats in the SKF-fed group being marginally heavier after 20 weeks of feeding (481 ± 15 g) than those in either the SSO-fed group (469 ± 11 g) or those receiving CC, the commercial rat chow (464 ± 13 g). These differences in mean body weight are not statistically significant at the 5% level.

The atrium:ventricle weight was determined in about half of the animals from all 3 dietary groups. No significant differences were found between dietary treatments and the ratio for all animals (mean ± SEM) is 0.11 ± .004.

The total phospholipid and cholesterol content of both the ventricle and atrium of rats receiving the different diets is given in Table 2. It is evident that there is no dietary-induced effect on either the total phospholipid or the total cholesterol content of these different components of the heart. It is also apparent that the cholesterol content of the atrium is always ca. 50% greater than that of the ventricle. The distribution of the major phospholipid classes of rat heart atrium and ventricle is shown in Table 3. PC comprises about 50% of the total phospholipids of both atrium and ventricle, PE is about 40% and DPG is about 7%. The remainder is accounted for as phosphatidylinositol (PI), phosphatidylserine (PS) and sphingomyelin (SPH), none of which separate readily from each other in the chromatographic system employed in this work. However, in preliminary determinations using a chromatographic separation, based on the procedure of Fine and Sprecher (24), it was found that these minor phospholipid components occur in approximately equimolar proportions. This distribution of phospholipids is not significantly different between atrium or ventricle, nor is it altered significantly either by an increase in the amount of dietary fat consumed or by widely different proportions of P/S in the diet.

The effect of diet on the fatty acid composition of

TABLE 2

Effect of Dietary Lipid Supplements on the Phospholipid and Cholesterol Content of Rat Heart

nmol/mg protein	CC		SSO		SKF	
	Ventricle	Atrium	Ventricle	Atrium	Ventricle	Atrium
Phospholipid	223±48	230±49	221±43	225±49	219±45	230±45
Cholesterol	14.8±1.3	22.1±2.0	14.2±0.3	23.3±1.9	15.7±1.2	22.1±2.4
Phospholipid/ cholesterol	15.0	10.0	15.6	9.6	13.9	10.4

CC = commercial rat chow; SSO = sunflower seed oil; SKF = sheep kidney fat

Values shown are the mean ± SEM for at least 5 determinations on separate samples of cardiac muscle from each dietary treatment.

TABLE 3

Distribution of Major Phospholipids in Ventricle and Atrium of Rats Fed Lipid Supplement Diets

Phospholipid Class	CC		SSO		SKF	
	Ventricle n = 9	Atrium n = 9	Ventricle n = 9	Atrium n = 9	Ventricle n = 11	Atrium n = 11
PC	49.4 ± 0.9	49.8 ± 1.6	48.7 ± 1.0	52.7 ± 1.2	49.3 ± 0.8	51.2 ± 1.1
PE	38.1 ± 0.7	37.4 ± 1.4	39.2 ± 0.9	36.3 ± 0.9	38.8 ± 1.2	35.0 ± 1.2
DPG	7.2 ± 0.9	5.9 ± 1.5	7.5 ± 0.9	6.0 ± 0.6	7.3 ± 0.5	6.4 ± 0.4

CC = commercial rat chow; SSO = sunflower seed oil; SKF = sheep kidney fat.

Diets CC, SSO and SKF are described in the text.

PC = phosphatidylcholine; PE = phosphatidylethanolamine; DPG = diphosphatidylglycerol. Values given are mean ± SEM of individual determinations on at least 9 separate muscle samples and are expressed as % of total phospholipid extracted from each tissue. The remainder was made up of small proportions of SPH, PI and PS which occurred in about equimolar proportions in all assays (see text). Differences between dietary groups or ventricles vs atria within a dietary group are not significant at 1% level ($p > .01$).

TABLE 4

Effect of Dietary Lipid supplements on the Fatty Acid Composition of Rat Heart PC

Fatty Acid (C:d, ω-x)	CC		SSO		SKF		LSD
	Ventricle n = 5	Atrium n = 5	Ventricle n = 7	Atrium n = 7	Ventricle n = 7	Atrium n = 6	
16:0 DMA	0.8 ± 0.1	0.8 ± 0.1	0.2 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	n.s.
16:0	17.6 ± 0.3	18.9 ± 0.5	11.3 ± 0.3	14.0 ± 0.3	13.5 ± 0.4	13.3 ± 0.5	2.3
17:0	0.5 ± 0.1	0.6 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	n.s.
18:0 DMA	---	0.5 ± 0.2	---	---	---	0.2 ± 0.1	n.s.
18:0	26.8 ± 0.4	26.5 ± 0.5	34.4 ± 0.4	32.9 ± 0.4	31.6 ± 10.4	32.9 ± 1.1	3.3
16:1	1.5 ± 0.1	2.6 ± 0.2	---	0.3 ± 0.2	1.0 ± 0.4	1.0 ± 0.6	1.9
18:1	10.6 ± 0.5	15.5 ± 0.4	7.2 ± 0.3	12.0 ± 0.4	10.1 ± 0.3	14.8 ± 0.2	1.9
18:2ω-6	12.4 ± 0.6	6.4 ± 0.4	8.7 ± 0.2	7.4 ± 0.7	6.3 ± 0.7	4.6 ± 0.3	3.0
18:3ω-3	---	0.2 ± 0.1	---	---	---	0.2 ± 0.1	n.s.
20:1	0.3 ± 0.1	1.0 ± 0.1	0.1 ± 0.1	0.4 ± 0.1	0.2 ± 0.1	0.6 ± 0.2	n.s.
20:3ω-6	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	n.s.
20:4ω-6	19.7 ± 0.8	16.2 ± 1.1	29.3 ± 0.3	23.8 ± 0.5	23.5 ± 0.7	20.1 ± 0.6	4.0
22:4ω-6	---	0.7 ± 0.1	0.4 ± 0.1	2.3 ± 0.1	---	0.5 ± 0.1	0.6
22:5ω-6	1.3 ± 0.1	1.4 ± 0.2	0.6 ± 0.1	0.6 ± 0.2	1.7 ± 0.1	1.6 ± 0.1	0.7
22:6ω-3	7.2 ± 0.2	4.0 ± 0.3	5.7 ± 0.2	3.3 ± 0.1	10.0 ± 0.3	5.4 ± 0.4	1.6

rat heart PC is given in Table 4. As shown, this particular phospholipid accounts for about half the total phospholipids of the cardiac membranes and is rich in the saturated fatty acids palmitic (16:0) and stearic (18:0), as well as containing significant amounts of both mono- and polyunsaturated acids. Characteristically, in PC, the polyunsaturates of the ω-6 family, linoleic (18:2) and arachidonic (20:4), exceed those of the ω-3 family, as docosahexaenoic acid (22:6) only occurs to ca. 5-10% of the total, and its precursor, linolenic acid (18:3), is virtually absent. PC contains a higher proportion of arachidonic acid than any other phospholipid found in the hearts of the rats examined in this study.

This pattern of fatty acid distribution (the fatty acid profile) is characteristic of cardiac PC and is similar, but not identical, in both ventricle and atrium. In general, the major saturated fatty acids, 16:0 and 18:0, do not differ greatly in proportion between ventricle and atrium, but the major polyunsaturated fatty acids, 18:2, 20:4 and 22:6, are all higher in ventricle than atrium, whereas the monounsaturated acids, 16:1, 18:1 and 20:1, are reduced.

Feeding lipid supplements of widely different levels of unsaturation for 20 weeks does not alter significantly the distribution between saturated and unsaturated fatty acids (ΣSAT:ΣUNSAT) in PC. However, the relative proportion of stearic acid (18:0) is increased, whereas that of palmitic (16:0) is decreased, by both lipid supplements employed in this study, when compared to the level found after feeding the control diet.

Although increasing the animals' intake of linoleic acid (18:2) by feeding SSO increases the proportion of arachidonic acid (20:4) in cardiac PC relative to the reference group (CC), there is a significant decrease in the proportion of ventricular 18:2 itself, presumably due to increased metabolic turnover of this EFA. Atrial arachidonate appears to rise

without any significant change in the level of its precursor. A reduction in 22:6 also occurs under these experimental conditions (SSO diet), probably since the ω-6 pathway for desaturation and elongation dominates that of the ω-3 family of fatty acids, and the relatively small proportion of 18:3 available in the diet is reduced even further by the addition of SSO (Table 1).

Feeding the more saturated SKF diet also results in an increase in the proportion of stearic acid (18:0), whereas that of palmitic decreases relative to the reference group (CC) but remains higher than that of the SSO fed group. The major reduction in dietary linoleic acid which occurs on feeding the SKF diet results in a significant fall in 18:2. This, however, is not accompanied by a reduction in 20:4, which remains at levels comparable to those found under control conditions. There is also a rise in 22:6, particularly in the ventricle, which can probably be attributed to the increased turnover of 18:3 which occurs when there is a reduction in the available 18:2. Thus, both the ventricle and the atrium of SKF-fed animals now contain relatively more 22:6 and less 20:4 and 18:2 than do those of the SSO fed group, i.e. there has been a shift in the ω-3/ω-6 ratio under these experimental conditions.

Like PC, rat cardiac PE has a characteristic fatty acid profile (Table 5). It is relatively rich in the saturated fatty acids (palmitic and stearic) which, as previously reported by Kramer (5), also generate appreciable amounts of their dimethylacetal derivatives (DMA) on methylation during analysis. Because of compensating changes in 16:0 DMA and 18:0 DMA, the total levels of these derivatives do not change on dietary lipid treatment with either SSO or SKF supplements.

Although the proportion of 18:1 in the atrium is consistently higher than in the ventricle, that of 18:2 is lower as the turnover of this acid seems to be increased, resulting in significantly increased levels of 20:4. Ventricular 22:6 is also consitently higher than that of the atrium, suggesting increased rates of turnover of both ω-6 and ω-3 fatty acids in the PE fraction of the ventricle.

Some important differences in the fatty acid profile of cardiac PE can be attributed to the diet. Feeding a linoleic acid-rich supplement (the SSO diet) results in a reduction in the proportions of docosahexaenoic acid (22:6) in both ventricle and atrium, but feeding a relatively low linoleic acid (22:6) supplement (the SKF diet) results in an increase in the proportions of the 22:6 long-chain polyunsaturated component of cardiac membranes.

TABLE 5

Effect of Dietary Lipid Supplements on the Fatty Acid Composition of Rat Heart PE

Fatty Acid (C:d, ω-x)	CC		SSO		SKF		LSD
	Ventricle n = 5	Atrium n = 5	Ventricle n = 7	Atrium n = 7	Ventricle n = 7	Atrium n = 6	
16:0 DMA	6.1±0.8	6.3±1.4	4.2±0.5	4.1±1.0	3.6±0.6	4.2±0.9	3.8
16:0	9.4±0.1	11.6±0.9	7.8±0.4	8.4±0.7	7.9±0.3	9.4±0.9	3.4
17:0	0.5±0.1	0.5±0.1	0.2±0.1	0.2±0.1	0.5±0.1	0.4±0.1	n.s.
18:0 DMA	2.3±0.1	2.3±0.9	4.3±0.3	4.2±0.8	5.3±0.4	5.6±0.6	2.8
18:0	24.9±0.5	23.4±0.9	25.0±0.6	24.7±1.8	25.1±0.4	22.3±0.7	n.s.
24:0	--	--	1.4±0.3	--	---	---	n.s.
16:1	1.3±0.2	2.2±0.7	0.8±0.3	1.5±0.6	1.0±0.3	2.5±0.6	n.s.
18:1	7.5±0.2	10.7±1.2	7.1±0.7	10.6±1.0	7.4±0.3	10.1±0.7	4.2
18:2ω-6	5.5±0.2	2.0±0.1	6.3±0.6	3.3±0.2	2.3±0.1	1.5±0.1	1.6
20:1	--	0.8±0.2	0.2±0.1	0.5±0.2	---	0.4±0.2	n.s.
20:4ω-6	11.8±0.2	17.3±1.3	14.7±0.3	21.2±1.7	12.9±0.4	19.2±1.1	5.8
22:4ω-6	0.4±0.1	1.6±0.1	1.3±0.2	5.5±0.5	0.1±0.1	1.3±0.2	1.3
22:5ω-6	1.9±0.1	2.4±0.2	1.0±0.1	1.0±0.1	1.9±0.1	2.6±0.2	0.9
22:6ω-3	26.8±0.7	13.9±0.8	22.8±0.9	10.6±0.6	30.1±0.6	16.4±1.2	4.5
Σ Saturates	45.7	47.3	46.1	47.5	46.0	47.3	
Σ Unsaturates	53.3	48.3	52.2	50.2	53.0	49.1	
Σω-3	7.2	4.2	5.7	3.3	10.0	5.6	
Σω-6	33.7	25.0	39.2	34.2	31.7	27.1	
20:4/22:6	2.7	4.0	5.1	7.2	2.3	3.7	

CC = commercial rat chow; SSO = sunflower seed oil; SKF = sheep kidney fat.

Values shown are % total fatty acids (mean ± SEM) where n = number of individual tissue samples examined. Assays were always in duplicate. -- represents detectable amounts of fatty acids less than 0.1%. Small amounts of other minor fatty acid components of uncertain identity have been omitted from the table. DMA is the dimethylacetal derivative from plasmalogens during methylation. The composition of the diets CC, SSO, and SKF are described in Table 1. LSD is the least significant difference obtained from the pooled error estimates of analysis of variance. Mean values within any one row which differ by more than the LSD are significantly different at the 5% level. n.s. = not significantly different.

Therefore, there is now a major difference between the 22:6 content of the PE fraction of the hearts of SSO- and SKF-fed rats. When this is considered in relation to the changes in arachidonic acid content which also occur on these diets, the ratio of 20:4/22:6 between SSO-fed rats and those of both the CC- and SKF-fed groups has changed significantly. Seemingly, an increase in dietary 18:2 enhances the turnover of ω-6 acids at the expense of the ω-3 family, whereas a reduction in 18:2 in the diet encourages the conversion of ω-3 acids. This is particularly evident in the PE fraction where the levels of 22:6 are much higher than in other phospholipid classes.

Although by comparison with either PC or PE, the DPG, or cardiolipin, content of rat hearts is low (c.f. Table 3), this phospholipid is of interest because it is extremely rich in the EFA linoleic acid (18:2, ω-6) which, in the ventricle, accounts for more than half of the total fatty acids present in this fraction (Table 6). The proportion of this essential polyunsaturated fatty acid in the ventricle is consistently higher than in the atrium (about 75%, compared to 50%), and this difference is maintained on feeding either a SSO or a SKF supplement. On the other hand, there is almost no conversion of 18:2 to 20:4 in the ventricular DPG fraction, although small but appreciable quantities of 20:4 are found in the atrium.

Similarly, the ventricle contains only small amounts of 22:6, whereas appreciable amounts of this product of ω-3 fatty acid metabolism are found in the atrium. However, it is apparent that feeding the low linoleic acid diet (SKF) again produces a significant change in the ratio of 20:4/22:6 in the DPG fraction of the ventricle of the rat.

DISCUSSION

Previous experiments in our laboratory have shown that atrial and ventricular tissues of the rat heart respond differently to dietary influences which may be related to differences in the phospholipid fatty acid composition of these cardiac muscles (8,9). In the present study, we examined this possibility in greater detail by determining both the relative proportions of the major classes of phospholipids and the fatty acid composition of these phospholipids in cardiac membranes. Dietary supplements of SSO and SKF were chosen to represent aspects of the diet of Western man, in general, and in particular, that of the Australian population where both SSO-based margarines and ruminant animal fats are common components of the diet (25). It was of interest to note that, despite the differences in the fat loading of the diet, both phospholipid and cholesterol contents of cardiac muscles were unchanged. In addition, the distribution between the major phospholipid classes (PC, PE and DPG) is not significantly different between

TABLE 6

Effect of Dietary Lipid Supplements on the Fatty Acid Composition of Rat Heart DPG

Fatty Acid (C:d, ω-x)	CC Ventricle n = 5	CC Atrium n = 5	SSO Ventricle n = 7	SSO Atrium n = 7	SKF Ventricle n = 7	SKF Atrium n = 6	LSD
16:0 DMA	0.5±0.1	0.4±0.2	—	0.5±0.3	—	0.3±0.1	n.s.
16:0	4.4±0.5	7.1±0.8	4.1±0.7	7.9±1.5	4.4±0.7	7.6±0.5	4.7
18:0 DMA	0.3±0.2	0.2±0.1	—	0.2±0.1	0.3±0.1	0.2±0.1	n.s.
18:0	3.9±0.6	3.7±0.5	3.9±0.3	4.8±0.6	4.0±0.7	5.9±0.7	n.s.
16:1	2.8±0.4	3.6±0.3	0.9±0.3	3.4±0.7	2.1±0.6	3.3±0.5	2.8
18:1	7.7±0.4	21.3±0.8	7.5±0.6	18.6±0.7	11.3±0.7	24.4±1.3	4.6
18:2ω-6	73.2±2.8	49.2±1.6	77.3±2.4	48.2±3.0	66.8±3.4	40.4±1.9	14.9
20:1	0.3±0.1	0.3±0.1	0.2±0.1	0.5±0.2	0.3±0.1	0.2±0.1	n.s.
20:3ω-6	0.8±0.1	2.2±0.1	0.8±0.1	2.1±0.2	2.0±0.1	2.3±0.1	0.5
20:4ω-6	0.6±0.2	4.3±0.3	1.3±0.1	5.7±0.4	1.8±0.1	5.6±0.4	1.4
22:5ω-6	—	0.3±0.1	—	0.6±0.1	0.2±0.1	0.6±0.1	n.s.
22:6ω-3	1.3±0.2	4.6±0.5	1.4±0.3	4.8±0.8	4.3±0.4	6.8±0.4	2.8
Σ Saturates	9.1	11.4	8.0	13.4	8.7	14.0	
Σ Unsaturates	86.7	58.8	89.4	83.9	88.8	83.6	
Σω-3	1.3	4.6	1.4	4.8	4.3	6.8	
Σω-6	74.6	56.0	79.4	56.6	70.8	48.9	
20:4/22:6	0.5	0.9	0.9	1.2	0.4	0.8	

CC = commercial rat chow; SSO = sunflower seed oil; SKF = sheep kidney fat.

Symbols and nomenclature as for Table 4. LSD is the least significant difference obtained from the pooled error estimates of analysis of variance. Mean values within any one row which differ by more than the LSD are significantly different at the 5% level. n.s. = not significantly different.

atrium or ventricle, nor is it altered significantly either by an increase in the amount of dietary fat consumed or by widely different proportions of P/S in the diet. This latter finding therefore confirms and extends an earlier report by Kramer (5), who also observed no effect of dietary lipids on the proportions of the major phospholipids of rat heart after feeding various plant oil supplements. These observations thus indicate that dietary lipid-induced changes in the fatty acid composition of cardiac phospholipids are due primarily to changes in the fatty acids of existing lipids, rather than to the synthesis of new phospholipid.

Although differences in the contractile (inotropic) response of atrial and ventricular (papillary) muscles are therefore not readily explained at this level of membrane composition, more important differences are found in the proportions of individual fatty acids in the various phospholipid classes. For example, we had previously reported that the proportion of total 18:2 ω-6 was always higher in the ventricle than in the atrium (9). This can now be explained by the much higher proportion of 18:2 in ventricular DPG than in the atrium, as well as the increased proportions of this EFA in ventricular PC and PE. Similarly, the increased total ventricular 22:6 that we had observed (9) can now be accounted for by higher proportions of this long-chain polyunsaturated fatty acid in both PC and PE, which are not offset by the finding that atrial DPG is richer in 22:6 than is the ventricle.

It is of interest that, in PC, ventricular 20:4 is higher than in the atria. In PE, the situation is reversed, ventricular 20:4 being lower than in the atria. In DPG, only very low levels of 20:4 are found in the ventricle where the proportion of 18:2 is very high indeed. Although much lower levels of 18:2 are found in the atria, there is clearly greater conversion to 20:4 in this muscle, as appreciable amounts of arachidonic acid are apparent. Thus, the different proportions of ventricular and atrial 20:4 reported previously (9) cannot be attributed to a simple difference in the arachidonic acid content of any single phospholipid class.

However, more general changes in the phospholipid fatty acid profile of cardiac muscle can be seen following changes in the nature of the dietary lipid intake. For example, despite the 5-fold difference in the stearic acid content of SSO and SKF supplemented diets (6% and 30%, respectively; c.f. Table 1), both lipid supplements resulted in a significant increase in the proportion of 18:0 in cardiac PC, whether in the ventricle or the atria, which was not seen in either cardiac PE or DPG. An equally unexpected result was the finding that feeding a linoleic acid-rich diet in the form of SSO did not result in a significantly increased level of 18:2 in any of the major phospholipid components of cardiac membranes when compared to the levels found after feeding the control diet. In fact, in PC, feeding SSO results in a decrease in the proportion of this acid, which probably accounts for the decrease in total ventricular 18:2 relative to the control reported previously (9). However, the fall in 18:2 in cardiac PC is accompanied by a highly significant increase in its major ω-6 metabolic product - arachidonic acid (20:4). In PE, feeding SSO does not result in a significant change in the level of 18:2, but the level of 20:4 also increases, whereas in DPG there is little conversion of 18:2 to 20:4 despite the very high proportion of 18:2 which is found in this phospholipid.

The complexity of these dietary-induced changes in cardiac phospholipid unsaturated fatty acid composition suggests that the processes of desaturaion and chain elongation that operate prior to their incorporation into membrane components (26,27), are neither uniform between ventricle or atrium, nor apparently are they identical for each class of phospholipid which makes up the cardiac membranes of the rat. Naughton has suggested that in liver and brain the metabolic pathways that are involved are subject to complex control which is most effective at the first step in the sequence of metabolism (28).

Experiments with radioactively-labeled fatty acid substrates, similar to those previously carried out by Mohrhauer and Holman (29) for rat liver, or a detailed analysis of the interactions between dietary fatty acid components of complex mixtures (30), would be required to confirm that competitive inhibition of fatty acid metabolism also occurs between the ω-6 and ω-3 families of fatty acids in the rat heart. However, there is little reason to doubt the existence of this general mechanism in this organ. Our results suggest that the sequential processes involved may occur at different rates in the ventricle and the atrium, similar to the differences reported to occur between other tissues of the rat (31).

Whether the differences in the proportions of unsaturated fatty acids and in the cholesterol contents of atrium and ventricles are of some functional significance in these anatomically distinct muscles of the rat heart cannot be established from the present study. However, since it is probable that differential effects also exist for phospholipase activity on differing classes of cardiac membrane phospholipids (32), it can be seen that both the supply and release of membrane precursors for prostaglandin biosynthesis could be different between the atrium and the ventricle, and could be subject to very complex dietary modulation. As various prostaglandins are reported to possess both positive or negative inotropic activity (33), it is not surprising that much more experimental work will be required before the potential extent of dietary manipulation of cardiac contractility by

this suggested mechanism can be evaluated. However, the possible central role of altered cardiac membrane composition and function in the etiology of myocardial arrhythmias (15,16, 34) recommends that this effort be continued.

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REFERENCES

1. Beare-Rogers, J.L., Nera, E.A., and Craig, B.M. (1972) Lipids 7, 46-50.
2. Beare-Rogers, J.L., Nera, E.A., and Craig, B.M. (1972) Lipids 7, 548-552.
3. Dewailly, P., Sezille, G., Nouvelot, A., Fruchart, J.C., and Jaillard, J. (1977) Lipids 12, 301-306.
4. Ruiter, A., Jongbloed, A.W., van Gent, C.M., Danse, L.H.J.C., and Metz, S.H.M. (1978) Am. J. Clin. Nutr. 31, 2159-2166.
5. Kramer, J.K.G. (1980) Lipids 15, 651-660.
6. Opstvedt, J., Svaar, H., Hansen, P., Pettersen, J., Langmark, F.T., Barlow, S.M., and Duthie, I.F. (1979) Lipids 14, 356-371.
7. Kramer, J.K.G., Farnworth, E.R., Thompson, B.K., Corner, A.H., and Treholm, H.L. (1982) Lipids 17, 372-382.
8. Dryden, W.F., Charnock, J.S., McMurchie, E.J., and Abeywardena, M.Y. (1982) Br. J. Pharmacol. 76, 183P.
9. Charnock, J.S., Dryden, W.F., McMurchie, E.J., Abeywardena, M.Y., and Russell, G.R. (1983) Comp. Biochem. Physiol. 71B, 47-52.
10. Charnock, J.S., Gibson, R.A., McMurchie, E.J., and Raison, J.K. (1980) Molec. Pharmacol. 18, 476-482.
11. Holloway, C.T., and Holloway, P.W. (1977) Lipids 12, 1025-1031.
12. McMurchie, E.J., Gibson, R.A., Abeywardena, M.Y., and Charnock, J.S. (1983) Biochim. Biophys. Acta 727, 163-169.
13. Ten Hoor, F., van de Graaf, H.M., and Vergroesen, A.J. (1973) in Recent Advances in Studies on Cardiac Structure and Metabolism (Dhalla, N.S., ed.) Vol. 3, pp. 59-72, University Park Press, Baltimore.
14. De Deckere, E.A.M., and Ten Hoor, F. (1980) Nutr. Metab. 24, 396-408.
15. Katz, A.M., and Messineo, F.C. (1981) Circ. Res. 48, 1-16.
16. Katz, A.M., and Messineo, F.C. (1981) Hosp. Prac. 16, 49-59.
17. Baghurst, K.I., and Baghurst, P.A. (1981) Trans. Menzies Found. 3, 139-160.
18. Skipski, N.V.P., and Barclay, M. (1969) Methods Enzymol., 14, 567-572.
19. Bowyer, D.E., and King, J.P. (1977) J. Chromatogr. 143, 473-490.
20. Macgee, J., Ishikawa, T., Miller, W., Evans, G., Steiner, P., and Glueck, C.J. (1973) J. Lab. Clin. Med. 82, 656-662.
21. Peterson, G.L. (1977) Anal. Biochem. 83, 346-356.
22. Colquohoun, D. (1971) in Lectures on Biostatistics, pp. 210-213, Clarendon Press, Oxford.
23. Warner, R.G., and Brewer, L.H., Jnr. (1972) Nutr. Requir. Lab. Anim. 10, 56-93.
24. Fine, J.B., and Sprecher, H. (1982) J. Lipid Res. 23, 660-663.
25. Baghurst, K.I., and Record, S.J. (1983) J. Food Nutr. 40, 1-15.
26. Mead, J.F. (1968) Prog. Chem. Fats Other Lipids 9, 159-192.
27. Klenk, E. (1972) in Current Trends in the Biochemistry of Lipids, pp. 41-48, Academic Press, London.
28. Naughton, J.M. (1981) Int. J. Biochem. 13, 21-32.
29. Mohrhauer, H., and Holman, R.T. (1963) J. Lipid Res. 4, 151-159.
30. Caster, W.O., Andrews, J.W., Mohrhauer, H., and Holman, R.T. (1976) J. Nutr. 106, 1809-1816.
31. Sinclair, A.J. (1975) Lipids 10, 175-184.
32. Van Den Bosch, H. (1980) Biochim. Biophys. Acta 604, 191-246.
33. Karmazn, M., Tuana, B.S. and Dhalla, N.S. (1981) Can. J. Physiol. Pharmacol. 59, 1122-1127.
34. Gudbjarnason, S. and Hallgrimsson, J. (1979) in Ischemic Myocardium and Antianginal Drugs (Winbury, M.M. and Abiko, Y., eds.), pp. 213-224, Raven Press, New York.

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Effect of Dietary Fats on Desaturase Activities and the Biosynthesis of Fatty Acids in Rat-Liver Microsomes

M.M. MAHFOUZ*, T.L. SMITH, and **F.A. KUMMEROW,** *Burnsider Research Laboratory, University of Illinois, Urbana, IL 61801*

ABSTRACT

Four groups of rats were fed diets containing 15% (w/w) high-oleic safflower oil (SFO, rich in *cis*-18:1 acids), a mixture of 80% partially hydrogenated soybean oil plus 20% corn oil (H + CO, rich in *trans*-18:1 acids), lard (L, rich in saturated fatty acids) and corn oil (Co, rich in 18:2ω6). Fatty acid composition of liver microsomes and activities of the Δ^5, Δ^6 and Δ^9 desaturases were determined. Microsomal Δ^6 desaturase activity and arachidonic acid were lower in the H + CO group compared with SFO of L. No difference was found in the Δ^5 or Δ^6 desaturase activity of CO and SFO groups. Thus, the oleic-acid level of the SFO diet had no effect on the metabolism of 18:2ω6. Fluorescent polarization studies, using *trans*-parinaric acid as a probe, showed no differences between the physical states of phospholipid vesicles made from lipids isolated from each group. We concluded that the *trans*-18:1 acids in partially hydrogenated soybean oil have a more inhibitory effect than saturated acids on EFA metabolism, even in the presence of adequate amounts of essential fatty acid.
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INTRODUCTION

In technological societies, the consumption of partially hydrogenated fats has been increasing. These fats contain significant quantities of various positional isomers of octadecenoic acids with *trans* and *cis* double-bond configurations (1-3). These unnatural acids are now found in human tissues at levels as high as 14% of the fatty acids of certain lipids (4). In vitro studies using liver microsomes from animals deficient in essential fatty acids (EFA) (5,6) have shown that positional isomers of *cis*- and *trans*-octadecenoic acids inhibit liver microsomal desaturation of 18:2ω6 and 20:3ω6. In in vivo experiments, the isomeric octadecenoic acids present in partially hydrogenated soybean oil (PHSO) were found to aggravate the symptons of EFA deficiency, probably through the inhibitory effect of these isomers on the conversion of 18:2ω6 to 20:4ω6 (7). Also, feeding rats diets containing PHSO in the presence of a low level of 18:2ω6 (18% of the minimum nutrient requirement) was shown to affect the metabolism of EFA with a consequent change in the polyunsaturated fatty acid (PUFA) pattern of liver and heart phospholipids (8).

Previous studies were conducted to assess the effect of *cis*- and *trans*-18:1 acids (present in partially hydrogenated vegetable oils (PHVO)) on the essential fatty acid metabolism in vitro or in vivo. These studies used liver microsomal fractions or tissues from animals that had been fed diets deficient in EFA (5,6) or that contained levels of 18:2ω6 lower than the minimum nutrient requirement (8). Alfin-Slater and Aftergood have suggested that *trans*-siomeric fatty acids have no adverse effects in animal models when an adequate amount of EFA is provided (9). This suggestion has been based largely on weight gain, longevity and reproductive performance data. However, little is known about the effect of isomeric 18:1 acids present in PHVO on the EFA metabolism when an adequate amount of 18:2ω6 is supplied in the diet.

The present nutritional experiments were designed to investigate the effects of dietary isomeric *trans*-18:1 acids (present in PHSO) and saturated fatty acids on the Δ^5 and Δ^6 desaturase activities in rats with sufficient EFA. Desaturase activities were measured in incubation reactions using the microsomal fraction of the liver. The fatty acid pattern was also analyzed, especially for the ω6 acids of the hepatic microsomes, in order to determine relationships between the synthesis of ω6 PUFA and the role of dietary *trans* and saturated acids in these metabolic processes.

In the present study, we used corn oil (CO), high-oleic safflower oil (SFO), a mixture of 80% partially hydrogenated soybean oil and 20% corn oil (H + CO) and lard (L). All the diets contained adequate amounts of 18:2ω6. The membrane fluidity of the microsomal phospholipid vesicles was also measured to correlate changes in desaturase activities with any alteration of the physical state of the vesicular membranes. A preliminary account of portions of this work has appeared elsewhere (10).

*To whom correspondence should be addressed.

MATERIALS AND METHODS

Amimals and Diets

Male weanling Sprague-Dawley rats, divided into 4 groups of 12 rats each, were fed a modified AIN-76 semipurified diet (11). Fifteen percent of the diet, by weight, was comprised of CO, SFO, L or a mixture of H + CO, for 6 weeks. The composition of the basal diet and the fatty acid composition of the fat supplements are shown in Tables 1 and 2. Food was prepared as needed and stored at 4C. The rats were fed fresh food daily with food and water provided ad libitum. The rats were housed individually in suspended cages with wire mesh bottoms. Room lighting consisted of 12-hr periods of light and dark. The rats were weighed weekly and were not fasted before being killed so that the maximum activities of the liver desaturases could be measured (12). The dietary treatments showed no significant effect on net body weight. All animals were sacrificed at ca. 9 a.m. on the day of the experiment.

Chemicals and Reagents

All cofactors, NADH, CoA (Lithium salt) and bovine serum albumin V fraction (essentially free of fatty acid), were obtained from Sigma Chemical Corporation (St. Louis, MO). All other chemicals were analytical grade. Palmitic, linoleic and eicosa-8,11,14-trienoic acids were obtained from NuChek Prep., Inc. (Elysian, MN). [$^{1\text{-}14}$C]-Palmitic acid (sp. act. 56 Ci/mol), [$^{1\text{-}14}$C] linoleic acid (sp. act. 51 Ci/mol) and [$^{1\text{-}14}$C] eicosa-8,11,14-trienoic acid (sp. act. 55 Ci/mol) were purchased from New Enagland Nuclear (Boston, MA).

Incubation Conditions and Assay of Desaturases

The liver microsomes were assayed for Δ^5, Δ^6, and Δ^9 desaturase activities as previously described (13). Each incubation in 1 ml of a 0.15 M KCL-0.25 M sucrose solution contained (in μmoles): ATP, 5.0; CoA, 0.25; NADH, 1.0; $MgCl_2$, 5.0; glutathione, 1.5; NaF, 45.0 nicotinamide, 0.5; phosphate buffer (pH 7.0), 100.0; and 2 mg protein of a microsomal suspension. The microsomal protein concentration was measured according to Lowry et al. (14). The microsomes were separated by centrifugation at 105,000 x *g* for 2 hr (5). One hundred nmol (containing an amount of ^{14}C labeled acid equivalent to 0.1 μCi) of palmitic or linoleic and 40 nmol eicosa-8,11,14-trienoic acid in the form of sodium salt bovine albumin complex (1 μg free fatty acid/11.5 μg bovine serum albumin) were used as substrates. Under these conditions, the enzymes were saturated by the substrates. The incubations were carried out for 20 min in a Dubnoff metabolic shaker at 37 C. The products of the reactions under the assay conditions as described were proportional to the 2 mg protein concentration and the 20 min reaction time.

TABLE 1

Composition of Semipurified Diets

Component	g/100 g
Casein[a]	20.0
Dextrose[b]	30.0
Cornstrach[c]	25.0
Cellulose[d]	5.0
Fat[e]	15.0
Mineral mix[f]	3.5
Vitamin mix[g]	1.0
DL-methionine[h]	0.3
Choline[i]	0.2

[a]Shamrock brand, Erie Casein Co., Erie, IL.
[b]Staleydex 333, A.E. Staley Co., Decatur, IL.
[c]Food grade, A.E. Staley Co., Decatur, IL.
[d]Solka-Floc, Brown Co., Berlin, NH.
[e]Corn oil, a mixture of 80% partially hydrogenated soybean oil plus 20% corn oil, lard and high-oleic acid safflower oil provided by Best Foods/CPC International, Union, NJ.
[f]AIN Mieral Mixture 76, ICN Nutritional Biochemicals, Cleveland, OH.
[g]AIN Mineral Mixture 76, ICN Nutritional Biochemicals, Cleveland. OH.
[h]ICN Nutritional Biochemicals, Cleveland, OH.
[i]Choline Bitartrate, ICN Nutritional Biochemicals, Cleveland, OH.

TABLE 2

Dietary Fatty Acid Composition

Diet[a]	CO	SFO	L	H + CO
Fatty acids[b]				
16:0	10.4	4.9	24.7	10.2
16:1	ND[c]	0.1	2.3	ND[c]
18:0	1.8	2.6	15.0	10.7
18:1 t[e]	ND[c]	ND[c]	0.8	35.1
18:1 c[e]	25.6	76.1	43.5	25.7[d]
18:2ω6	61.3	15.2	10.0	15.3
18:3ω3	0.9	ND[c]	0.3	0.3
20:0	ND[c]	0.5	0.2	0.3
20:1ω9	ND[c]	0.3	0.6	0.1
20:2ω6	ND[c]	ND[c]	0.4	ND[c]
22:0	ND[c]	0.4	ND[c]	0.3
24:0	ND[c]	0.2	ND[c]	0.2
Others	0.0	0.0	2.2	1.8

[a]Semipurified diet containing 15% fat as corn oil (CO), high-oleic safflower oil (SFO), Lard (L) or a mixture of 80% partially hydrogenated soybean oil + 20% corn oil (H + CO).
[b]Carbon number: number of bonds, ω = double bond position from the methyl end.
[c]ND = not detected (<0.1%).
[d]This value represents a mixture of 80% *cis* 18:1ω9 + 20% of other *cis* isomers.
[e]c = *cis*, t = *trans*.

The reaction was terminated by the addition of 5% HCl in methanol and the lipids were extracted with chloroform/methanol (2:1, v/v). The extract was dried under a stream of N_2 and transesterified with 3 N methanolic-HCl (Supelco, Inc., Bellefonte, PA) at 70 C for 2 hr (7). The HCl-methanol was evaporated under N_2. The methyl esters were dissolved in petroleum ether. A mixture of unlabeled carriers of methyl esters of 16:0 + 16:1, 18:2 + 18:3 or 20:3 + 20:4 were added to the incubation products. The esters were separated on 10% $AgNO_3$ Silica Gel H plates as previously described (6). The separated bands were scraped into scintillation vials, 10 ml of scintillation fluid (4 gm PPO + 84 mg POPOP/l toluene) was added, and the activity was counted in a Packard Scintillation Counter. The percentage of desaturation was calculated as the ratio of the counts in the desaturated products to the sum of the counts in the substrate plus product, corrected for background. The nmol of the product were then calculated. The recovery of the radioactivity was more than 85% of the amount used.

Extraction of Lipids and Analysis of Fatty Acids

In order to study the effect of the different dietary treatments on the fatty acid composition of the total lipids and the phospholipids of the liver microsomal fractions, the microsomes were extracted according to the Folch method (15) and the lipid extract was divided into 2 portions. One portion was used for preparing the methyl ester of the total lipids following saponification and methylation (16). The phospholipid fraction was seaprated from the other portions of the lipid extract by polysilicic acid gel-impregnated glass fiber sheets (ITLC), using a solvent system of petroleum ether/diethyl ether (90:10, v/v). The phospholipids were transesterified by BF_3-methanol complex (17). BHT was added as antioxidant in a concentration of 0.005% (w/v) to all the solvents used for lipid extraction and chromatography. All operations were carried out under N_2.

A Packard Model 428 gas chromatograph (Hewlett Packard Instrument Co., Inc., Chicago, IL), equipped with an all-glass injection splitter and a flame ionization detector (FID) was used to separate the methyl esters on a Quadrex, 60 m × 0.25 mm ID SP 2340 wall-coated, open-tubular (WCOT) glass column (Quadrex Inc., New haven, CT). The oven temperature was programmed from 160-220 C at 1 C/min and the injector and detector temperatures were 280 C and 300 C, respectively. The N_2 flow rate was 0.7 ml/min with a split ratio of 110:1. Retention time, peak areas and peak relative area percentages were determined electronically using a Hewlett Packard Model 3390A Reporting Integrator. Identification of methyl esters of fatty acids was accomplished by comparing relative retention times with authentic standards (NuChek Prep., Elysian, MN, and Supelco, Inc., Bellefonte, PA). A test of statistical significance was applied using a one-way analysis of variance with mean separation by Duncan's multiple range test (18).

Preparation of Microsomal Phospholipid Vesicles and Fluorescence Measurement

The microsomes isolated above were subjected to a modified Bligh and Dyer extraction (19). To 0.8 ml of a concentrated suspension of microsomes was added 3 ml chloroform/methanol (1:2, v/v), which was vortexed and then centrifuged at 200 × *g* to precipitate the residue. The supernatant was saved and the residue reextracted with 1 ml of chloroform. After centrifugation, the chloroform was collected with the previous supernatant. To the combined supernatants, 1 ml 0.1 M KCl was added. After centrifugation, the upper layer and any interfacial material was discarded. The extracts were dried, weighed and suspended in 1 ml chloroform.

Phospholipids were isolated by Unisil (activated silicic acid 200-325 mesh) chromatography. For each mg of lipid, 0.1 g of acid-washed, activated Unisil was put in a gooch funnel (minimum of 0.5 g) and washed with 5 ml of chloroform. The sample was applied in chloroform to the Unisil and then wased 3 times with 5 ml portions of chloroform to remove any neutral lipids. The phospholipids were eluted with 5 ml of methanol. Methanol was removed under vacuum with chloroform added to reduce the boiling point. The dry lipid was dissolved in 2 ml chloroform and the phospholipid molar concentration determined using ammonium ferrothiocyanate (20) with egg yolk phosphatidylcholine as a standard.

trans-parinaric acid was obtained from Molecular Probes (Junction City, OR) and stored in ethanol with .001% BHT at -20 C in the dark under N_2. To make vesicles, 1.2 μmol phospholipid were transferred to a screw-cap test tube and dried in a thin film on the side of the tube under a stream of N_2. Six ml potassium phosphate buffer (50 mM, pH 7.2) were added, the tube closed under N_2 and heated to 65 C. The tube was then vortexed at full speed for 15 sec and allowed to cool. Three ml were set aside for use as a scattering blank. To the remaining 3 ml was added 3 nmol of *trans*-parinaric acid (probe-to-lipid ratio of 1/200), then the tube was incubated at 37 C for 40 min.

Fluorescence polarization studies were done on a Perkin-Elmer spectrofluorimeter, model 650-10S (Norwalk, CT), fitted with a Hitachi model 650-0139 thermostatted turret cell holder, cell stirrer and polaroid filter rotator (C.N. Wood Manufacturing, Newtown, PA). Excitation occurred at 325 nm. Emission was read at 420 nm and fluorescence intensity measured for parallel ($I_{\parallel}$) and perpendicular ($T_{\perp}$) orientations of the emission filter. The polarization ratio was calculated as $(I_{\parallel}-I_{\parallel}s)/((I_{\perp}-I_{\perp}s)\times G)$, where $I_{\parallel}s$ and $I_{\perp}s$ are corrections for scattering and G is the grating factor equal to HH/HV (21). Readings were made while heating the cuvette from below the lowest temperature to the highest. Heating rates were less than 1 C/min.

RESULTS AND DISCUSSION

The percentage of the components of the dietary fatty acids used in this study are shown in Table 2. SFO, L and H + CO diets contained about 82% of their total fatty acids as 18:1 plus saturated fatty acids. The SFO diet contained 76% of the total fatty acids as *cis* Δ^9 18:1 and 7.5% as saturated fatty acids (16:0 + 18:0). The H + CO diet contained 60.8% as 18:1 acids and 20.9% saturated acids. The 60.8% present as 18:1 acids was distributed as 25.7% 18:1 *cis*-isomers (80% *cis*-Δ^9 18:1 and 20% other 18:1 isomers) and as 35.1% *trans*-18:1 isomers. SFO and H + CO diets are clearly high in 18:1 acids, but they are different in the double-bond configuration of these 18:1 acids. *Trans*-18:1 acids are more like saturated fatty acids than *cis*-18:1 acids in their structure and melting point. The lard fat, which contained 40% saturated fatty acids and 43% *cis*-Δ^9 18:1 acid, was used as a control for the effect of saturated fatty acids. These diets allow comparison between the effect of *trans*-18:1 acids and the effect of saturated fatty acids on the linoleic acid metabolism when this acid is present in sufficient amounts because these 3 diets contained adequate amounts of 18:2ω6 at comparable levels.

The data listed in Table 3 shows the fatty acid profile of the liver microsomal lipids from rats fed CO, SFO, L or H + CO diets. Following a 6-week diet containing 15% fat, of which 35% were *trans*-18:1 acids, the amount of *trans*-18:1 acids deposited in the microsomal lipids of the H + CO group reached 12.8%. The presence of sufficient amounts of 18:2ω6 in the H + CO diet used in the present study did not prevent the uptake of the *trans*-acids into the microsomal lipids (8).

The total saturated fatty acids (16:0 + 18:0) supplied in the H + CO diet (20.9%) was much higher than the amount supplied in the SFO diet (7.5%) (Table 2), but the total amount of

TABLE 3

The Effect of Dietary Fats on Rat-Liver Microsomal Tatal Lipid Composition

Diet[+]	CO (n=12)	SFO (n=12)	L (n=12)	H + CO (n=12)
Fatty acids*				
16:0	17.3 ± 0.0[c]	16.7 ± 0.53[b,d]	18.3 ± 0.63[a,b]	13.5 ± 0.61[a,c,d]
16:1 ω7	0.7 ± 0.1[++]	0.7 ± 0.07	1.1 ± 0.30	1.0 ± 0.33
18:0	17.0 ± 0.8[b,d,e]	19.2 ± 0.34[c,d]	20.5 ± 0.50[a,b]	13.9 ± 0.22[a,c,e]
18:1 t	—	—	—	12.8 ± 0.70
18:1 c	6.8 ± 0.4[a,d,f]	15.3 ± 1.0[a,b,c]	11.8 ± 0.53[c,d,e]	9.6 ± 0.50[b,e,f]
18:2c,t + t,c	—	—	—	0.3 ± 0.01
18:2 ω6	15.2 ± 0.7[a,b,c]	6.1 ± 0.36[a,d]	6.3 ± 0.62[b,e]	10.3 ± 0.57[c,d,e]
18:3 ω6	0.3 ± 0.01	0.2 ± 0.02	0.1 ± 0.00	0.3 ± 0.04
20:2 ω6	1.0 ± 0.1	0.3 ± 0.03	0.2 ± 0.03	0.2 ± 0.02
22:0 + 20:3ω9	0.4 ± 0.02	0.7 ± 0.05	1.0 ± 0.12	0.3 ± 0.03
20:3 ω6	0.6 ± 0.06[a]	0.6 ± 0.03[b]	0.8 ± 0.12	0.9 ± 0.10[a,b]
20:4 ω6	29.8 ± 0.5[b]	30.5 ± 0.38[a]	29.0 ± 0.74[c]	24.6 ± 0.40[a,b,c]
22:4 ω6	1.3 ± 0.1[a,b,c]	0.8 ± 0.06[c,d,e]	0.6 ± 0.05[b,e]	0.5 ± 0.02[a,d]
22:5 ω6	2.5 ± 0.3[c]	4.3 ± 0.22[a,b,c]	2.4 ± 0.26[b]	1.8 ± 0.30[a]
22:5 ω3	0.5 ± 0.05	—	0.4 ± 0.04	0.3 ± 0.02
22:6 ω3	3.3 ± 0.2[c,d]	1.5 ± 0.01[a,d,e]	4.0 ± 0.38[a,b,c]	3.2 ± 0.10[b,e]
20:4	0.4 ± 0.1	0.5 ± 0.04	0.4 ± 0.08	0.3 ± 0.02
Other		2.6	3.1	6.1

t = *trans*, c = *cis*.

[+]Animals fed 15% corn oil (CO), high-oleic safflower oil (SFO), lard (L) or a mixture of 80% partially hydrogenated soybean oil + 20% corn oil (H + CO).

*All fatty acids are of the *cis* configuration unless otherwise noted.

[++]Mean ± SEM. Means within the same line with a superscript letter in common are statistically different at the $p<0.05$ level, as tested by Duncan's multiple-range test procedure.

saturated fatty acids that accumulated in the microsomal lipids of the H + CO group (27.4%) was lower than in the SFO group (35.9%). This can be attributed to the accumulation of the *trans*-18:1 acids by the microsomes of the H + CO group, which compete with the saturated fatty acids at the α-position of the acyl lipids. Also, the proportion of saturated fatty acids in the microsomes (including *trans*-18:1 acids) of rats fed different diets did not vary widely (Table 3), although the quantity taken in was different, depending on the diet given (Table 2) (22).

Table 4 shows the distribution of ω6 PUFA in the liver microsomes of the SFO, L or H + CO group. The content of 18:2ω6 (the substrate for Δ^6 desaturase) was the same in the microsomes of the SFO and L groups ($p>0.05$) but increased significantly in the microsomes of the H + CO group compared with the SFO or L groups. The 20:4ω6 acid, which is considered the major product in the synthesis of ω6 PUFA in the microsomal lipids, decreased significantly in the H + CO group compared with the L or SFO groups, whereas no significant difference was observed between the L and SFO groups.

The amount of 22:5ω6 acid was significantly less in the microsomes of the L and H + CO groups than the SFO group ($p<0.05$). The decrease of 22:5ω6 acid in the microsomes of the L and H + CO groups can be related to the inhibitory effect of 22:6ω3, which was present in significantly higher amounts in the microsomes of the L (4.0%) and H + CO (3.2%) groups than in the SFO (1.5%) group (Table 3). The 22:6ω3 acid, which was present at a higher level in the microsomes of the L and H + CO groups, was not present in the diets fed the those groups (Table 2), so it must be derived from the desaturation and elongation of 18:3ω3 acid, which was 0.3% of the fatty acids of the L and H + CO diets (Table 2). This observation is consistent with the higher affinity of the Δ^6 desaturase for 18:3ω3 compared with 18:2ω6 reported by Brenner (23). However, the conversion of linoleic acid to arachidonic acid in the microsomes of the L group was not significantly different from the SFO group ($p>0.05$) (Table 4). This result was expected given that 18:3ω3, as substrate for the same enzymes, was present in a very small amount (0.3%) compared with 18:2ω6 acid (10%) in the L diet.

The small but significant decrease in the total amount of ω6 acids in the microsomes of the L group compared with the SFO group must be attributed to the lower content of dietary 18:2ω6 supplied to the L group (10%) compared with the SFO group (15%). Although the amount of dietary 18:2ω6 supplied to the SFO and H + CO groups was the same (15%), the amount of ω6 acids incorporated into the microsomal lipids of the H + CO group was significantly less than in the SFO group. The low level of total ω6 acids in the H + CO group can be related to the presence of *trans*-18:1 acids in the microsomes of this group as the amount of ω6 acids in liver phospholipid decreased as the amount of *trans*-acids increased (8). The amount of ω6 metabolites, which represent the desaturation and chain elongation products of 18:2ω6, was significantly less in the microsomes of the L group than in the SFO group; however, as mentioned before, this decrease is caused by the lower dietary level of

TABLE 4

ω6 Polyunsaturated Fatty Acids Distribution and the Ratios Between the ω6 Metabolits in Liver Microsomes of Rats Fed SFO, L or H + CO Diets

Diet[+]	SFO	L	H + CO
Fatty acids			
18:2 ω6	6.1 ± 0.36[a]	6.3 ± 0.62[b]	10.3 ± 0.57[a,b]
18:3 ω6	0.2 ± 0.02*	0.1 ± 0.00	0.3 ± 0.04
20:2 ω6	0.3 ± 0.03	0.2 ± 0.03	0.2 ± 0.02
20:3 ω6	0.6 ± 0.03[a]	0.8 ± 0.12	0.9 ± 0.10[a]
20:4 ω6	30.5 ± 0.38[a]	29.0 ± 0.74[b]	24.6 ± 0.40[a,b]
22:4 ω6	0.8 ± 0.06[a,b]	0.6 ± 0.05[b]	0.5 ± 0.02[a]
22:5 ω6	4.3 ± 0.22[a,b]	2.4 ± 0.26[b]	1.8 ± 0.30[a]
Total ω6 acids	42.8 ± 0.29[a,b]	39.4 ± 0.70[b]	38.4 ± 0.65[a]
Total ω6 metabolites	36.7 ± 0.46[a,b]	33.1 ± 0.57[b,c]	28.1 ± 0.41[a,c]
(ω6 metabolites)/(ω6 acids)	0.86 ± 0.01[a]	0.84 ± 0.01[b]	0.73 ± 0.01[a,b]
20:4ω6/18:2ω6	5.0 ± 0.31[a]	5.2 ± 0.17[b]	2.4 ± 0.11[a,b]

—Results at the same line with common superscript are statistically different from each other for $p<0.05$ as tested by Duncan's multiple-range procedure.

[+]Animals fed 15% high oleic safflower oil (SFO), lard (L) or a mixture of 30% partially hydrogenated soybean oil + 20% corn oil (H + CO).

*Mean ± SEM.

18:2ω6 supplied to the L group. Yet, even though the H + CO group was supplied with the same level of dieatry 18:2ω6 as the SFO group, which was higher than that of the L group, the amount of ω6 metabolites in the microsomes of the H + CO group was still significantly lower than in the L and the SFO groups (Table 4).

The ratio of (ω6 metabolites)/(ω6 acids) was significantly lower in the H + CO group than in the SFO or L groups, but no significant difference was observed between the L and SFO group ($p>0.05$). The conversion of 18:2ω6 to 20:4ω6, which involves Δ^6 desaturation, chain elongation and Δ^5 desaturation, was measured by the ratio of 20:4ω6/18:2ω6 (24). This ratio was significantly decreased in the H + CO group compared with the L or SFO groups. Again, no significant difference was found between the L and SFO groups ($p>0.05$).

The results from the in vitro measurements of Δ^6, Δ^5, and Δ^9 desaturase activities in liver microsomes from rats fed the different experimental diets are presented in Table 5. The rats in the present study were subjected to controlled lighting conditions and were killed at a fixed time to reduce the influence of possible diurnal changes on the measured enzyme activities (25). The Δ^6 desaturase activity in the microsomes of the L group was lower than in the SFO group, but this decrease was not significant ($p>0.05$). In the microsomes of the H + CO group, the Δ^6 desaturase activity was significantly lower than in the SFO or L groups. The in vivo effects of the *trans*-18:1 acids present in the H + CO diet on the conversion of 18:2ω6 to 20:4ω6, as well as on the distribution of ω6 PUFA in the microsomal lipids (Table 4), do correlate with the microsomal desaturase activities obtained in vitro. The accumulation of 18:2ω6, the substrate of Δ^6 desaturase and the decreased level of 20:4ω6 observed in the microsomal lipids of the H + CO group compared with the SFO group, are consistent with the tendency toward decreased microsomal Δ^6 desaturase activity obtained in vitro (Table 5).

The Δ^5 desaturase activity was slightly lower in the microsomes of the L and H + CO groups than in the SFO group, but no significant differences were observed between groups (Table 5). The 20:3ω6 acid, the substrate for Δ^5 desaturase, was also slightly higher in the microsomes of the L and H + CO groups than in the SFO group (Table 4). Undetectable differences in the rate of reaction over extended time may lead to larger, more easily detectable differences in the products incorporated and accumulated in structural lipids (8).

The Δ^5 desaturase activities obtained in vitro were higher than those of Δ^6 desaturase (23), as shown in Table 5. Consequently, the Δ^6 desaturase is likely to be the main regulatory enzyme in the synthesis of PUFA.

Although the Δ^9 desaturase is not directly involved in the metabolism of ω6 PUFA, it was included for comparison. No significant differences were observed for Δ^9 desaturase activity among the SFO, L and H + CO groups (Table 5). The amount of monounsaturated fatty acids (16:1 + 18:1) incorporated into liver microsomal lipids (Table 3) are more or less proportional to the amounts provided in the diets (Table 2). In a previous report, we found that the positional isomers of *trans*-18:1 acid inhibit liver microsomal Δ^9 desaturase (5). This previous study, however, was carried out in vitro and used liver microsomes of EFA-deficient rats. In a recent study, Svensson (26) showed that Δ^9 desaturase activity was higher in the liver microsomes of rats fed partially hydrogenated oils than in the control group. In his study, the control diet contained a high proportion of linoleic acid (37.8%) whereas the experimental, *trans*-containing diets contained a lower proportion of linoleic acid (11%). The high content of linoleic acid in the control diet tends to decrease Δ^9 desaturase activity in rat-liver microsomes, as reported by Jeffcoat and James (27). In the present study, the SFO, L and H + CO diets contained comparable levels of 18:2ω6, whereas the microsomal lipids of each group contained equivalent levels of 18:2ω6 and 20:4ω6 (acids that have been shown to inhibit the Δ^9 desaturase) (27,28).

The possibility cannot be excluded that the large proportion of oleic acid (76%) in the SFO diet could inhibit the synthesis of ω6 PUFA. Mohrhauer et al. (29) have shown that dietary oleic acid, fed in proportions as high as 22 cal % of the diet, did not significantly alter the conversion of 18:2ω6 to 20:4ω6 if 18:2ω6 was fed at or above the minimal dietary requirement of ca. 1 cal %. Shimp et al. (30) also indicated that oleic acid had little impact on the conversion of 18:2ω6 to 18:3ω6 when added as an inhibitor in a concentration of 400 nmol to 50 nmol of 18:2ω6 as substrate in vitro. However, in the present study, we included another group of rats, that had been fed 15% corn oil (CO), to clarify this point. Table 3 shows a comparison among the fatty acid composition of the microsomal lipids of SFO, L and CO groups. As shown in Table 3, the level of arachidonic acid did not increase in the liver microsomal lipids of the CO group, which was fed a diet containing 61% 18:2ω6, compared with the SFO or L groups, which

were fed 15% and 10% 18:2ω6 in their diet. This finding indicates that the CO diet supplied more than an adequate amount of 18:2ω6 to meet the requirement for EFA. Evidence of this adequacy is supplied by the fact that the levels of arachidonic acid in the microsomal lipids of the SFO, L and CO groups were essentially the same (ca. 30%), as seen in Table 3. Kurata and Privett (31) and Tahin et al. (32) made the same observation when they fed 2 groups of rats 2 different levels of adequate amounts of 18:2ω6.

The CO group was also supplied with an amount of dietary 18:3ω3 3 times the amount supplied to the L group (Table 2), but the amount of 22:6ω3 acid derived from the desaturation and elongation of 18:3ω3 and accumulated in the liver microsomal lipids of the CO group (3.3%) was significantly lower than in the L group (4%). Thus, diets rich in linoleic acid not only induce an increase in ω6 PUFA but also inhibit the metabolism and incorporation of ω3 acids into the liver microsomes (32). This supports the observation by de Schrijver and Privett (33) that the accumulation of 22:6ω3 is under metabolic control and that a high quantity of ω6 long-chain fatty acids in the microsomes seems to limit 22:6ω3 incorporation.

Table 5 shows that Δ^6 and Δ^5 desaturase activities were not significantly changed in the CO group compared with the SFO or L groups. The Δ^9 desaturase activity was significantly decreased in the CO group compared with the SFO group, possibly because of the high level of dietary 18:2ω6 in the CO diets, which can affect Δ^9 desaturase (27). The high oleic acid in the SFO diet used in the this study did not significantly affect the linoleic acid metabolism, probably because of the higher affinity of Δ^6 desaturase for 18:2ω6 compared with *cis*-18:1ω9 (23).

In order to determine whether changes in desaturase activity were related to alterations in the physical state of the microsomal membrane caused by changes in the microsomal phospholipid fatty acids between dietary treatments, we isolated the microsomal phospholipids which were then used for preparing vesicles. The vesicular membrane fluidity was measured using *trans*-parinaric acid as a fluorescent probe. The fatty acid composition of the liver microsomal phospholipids of the different groups are shown in Table 6, and the fluorescent polarization ratio results are shown in Table 7. With *trans*-parinaric acid, a polarization ratio of less than 1.6 indicates that no solid lipid domain is present (34). The lack of solid domain, even at 5 C, correlates well with the ratio of the double-bond index to saturated fatty acids of ca. 5 for all diets, as shown in Table 6 (35). To determine whether any difference exists between the diets, the polarization ratio were evaluated by the F test at each temperature. The results are shown in Table 8. No differences were found between diets at any temperature. This demonstrates that the physical state of lipids derived from liver microsomal fractions of rats fed different diets was essentially the same. Thus, any differences in desaturase activity cannot be attributed to differences in membrane fluidity.

From this study, we can conclude that the *trans*-18:1 acids in partially hydrogenated soybean oil can affect the metabolism of essential fatty acids even in the presence of adequate amounts of dietary 18:2ω6. Acyl-CoA desaturases are membrane-bound enzymes; lipid cofactors are important for the maximum activity of these desaturases (36.37). Brenner (23) also suggested that lipoporteins play a specific role in the Δ^6 desaturation. Therefore, the *trans*-18:1 acids may possibly affect the integrity of a lipoportein structure or the alteration of the composition of lipid cofactors required for maximum desaturation. Further studies are needed to determine the specific action of *trans*-18:1 acids in the inhibition of

TABLE 5

In vitro Desaturase Activities of Liver Microsomes Derived from Rats Fed CO, SFO, L or H + CO Diets

Diet	CO (n=12)	SFO (n=12)	L (n=12)	H + CO (n=12)
Δ^6 desaturase (18:2ω6 → 18:3ω6)	0.29 ± 0.02[b]	0.34 ± 0.03[a]	0.28 ± 0.03[c]	0.20 ± 0.02[a,b,c]
Δ^5 desaturase (20:3ω6 → 20:4ω6)	0.55 ± 0.03	0.52 ± 0.03	0.45 ± 0.02	0.46 ± 0.03
Δ^9 desaturase (16:0 → 16:1ω7)	0.40 ± 0.03[a]	0.55 ± 0.02[a]	0.52 ± 0.09	0.45 ± 0.05

Results (mean ± SEM) are expressed as nmol of substrate converted per min per mg protein.

Results at the same line with a common supercript are statistically different from each other for $p<0.05$ as tested by Duncan's multiple-range test procedure.

TABLE 6

Fatty Acid Composition of Liver Microsomal Phospholipids from Rats Fed SFO, L or H + CO Diets

Diet[+]	SFO (n=6)	L (n=6)	H + CO (n=6)
Fatty acids*			
16:0	15.3 ± 0.28[b,c]	17.8 ± 0.68[a,b]	11.7 ± 0.42[a,c]
16:1 ω7	0.3 ± 0.02[++]	0.7 ± 0.16	0.6 ± 0.14
18:0	22.3 ± 0.73[b]	23.1 ± 0.40[a]	15.9 ± 0.55[a,b]
18:1 t	—	—	12.2 ± 0.4
18:1 c	10.2 ± 0.43[a,b]	8.1 ± 0.29[b,c]	6.9 ± 0.4[a,c]
18:2 c,t + t,c	—	—	0.2 ± 0.00
18:2 ω6	5.6 ± 0.41[a]	5.7 ± 0.60[b]	10.9 ± 0.46[a,b]
18:3 ω6	0.1 ± 0.01	0.1 ± 0.00	0.3 ± 0.03
20:2 ω6	0.4 ± 0.04	0.3 ± 0.05	0.4 ± 0.03
22:0 + 20:3ω9	0.9 ± 0.10	1.1 ± 0.17	0.8 ± 0.05
20:3 ω6	0.6 ± 0.03[a]	0.8 ± 0.13	1.0 ± 0.14[a]
20:4 ω6	32.7 ± 0.62[a]	30.9 ± 0.7[b]	27.0 ± 0.5[a,b]
22:4 ω6	1.0 ± 0.04[a,b]	0.7 ± 0.08[b]	0.6 ± 0.02[a]
22:5 ω6	5.1 ± 0.20[a,b]	2.5 ± 0.3[b]	1.9 ± 0.33[a]
22:5 ω3	0.1 ± 0.00[a,b]	0.3 ± 0.06[b]	0.3 ± 0.04[a]
22:6 ω3	1.5 ± 0.12[a,b]	4.9 ± 0.4[a]	4.2 ± 0.14[b]
24:0	0.6 ± 0.02	0.5 ± 0.05	0.4 ± 0.04
Other	3.3	2.5	4.7
Double bond index	5.0 ± 0.11[a]	4.7 ± 0.08	4.6 ± 0.10[a]
Saturated fatty acids**			

Results at the same line with a common superscripts are statistically different from each other at $p<0.05$ level as tested by Duncan's multiple-range test procedure. t=*trans*, c=*cis*.

[+]Animals fed 15% high-oleic safflower oil (SFO), lard (L), or a mixture of 80% partially hydrogenated soybean oil + 20% corn oil (H + CO).

*All fatty acid are of *cis* configuration unless otherwise noted.

[++]Mean ± SEM.

**_Trans_-18:1 acids were considered as saturated acid.

TABLE 7

Fluorescent Polarization Ratio of the Vesicular Membranes Prepared from the Liver Microsomal Phospholipids

Diet[a]	Polarization ratio (P)[b] SFO	L	H + CO
Temperature			
5	1.3	1.4	1.4
15	1.3	1.3	1.3
25	1.1	1.2	1.2
35	1.0	1.1	1.1

[a]Animals fed 15% high-oleic safflower oil (SFO), lard (L) or a mixture of 80% partially hydrogenated soybean oil + 20% corn oil (H + CO).

[b]Polarization ratio calculated as described in Methods. Values are the mean of at least 2 and generally 3 determinations.

TABLE 8

Statistical Evaluation of Polarization Ratios at Each Temperature

Temperature	F value[a]	Significance P>[b]
5	0.29	0.75
15	0.06	0.95
25	0.03	0.97
35	0.08	0.92

[a]F value = treatment mean squares/error mean squares.

[b]No difference in polarization ratio value at any temperature.

Δ^6 desaturase under EFA-sufficient conditions and to determine if the changes are of such magnitude that they are able to affect prostaglandin synthesis.

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REFERENCES

1. Schofield, C.R., Davison, V.L., and Dutton, H.J. (1967) J. Am. Oil Chem. Soc. 44, 648-651.
2. Carpenter, D.L., and Slover, H.T. (1973) J. Am. Oil Chem. Soc. 50, 372-376.
3. Reichwald, H.I., Ilsemann, K., and Mukherjee, K. (1979) J. Nutr. 109, 1051-1056.
4. Kummerow, F.A. (1974) J. Am. Oil Chem. Soc. 51, 255-259.
5. Mahfouz, M., Johnson, S., and Holman, R.T (1980) Lipids 15, 100-107.
6. Mahfouz, M., Johnson, S., and Holman, R.T. (1981) Biochim. Biophys. Acta 663, 58-68.
7. Hill, E.G., Johnson, S., and Holman, R.T. (1979)

J. Nutr. 109, 1759-1765.
8. Hill, E.G., Johnson, S., Lawson, L.D., Mahfouz, M., and Holman, R.T. (1982) Proc. Natl. Acad. Aci. USA 79, 953-957.
9. Alfin-Slater R.B., and Aftergood, L. (1979) in Geometrical and Positional Fatty Acids Isomers (Emken, E.A., and Dutton, H.J., eds.) pp. 53-74, Am. Oil Chem. Soc., Champaign, IL.
10. Mahfouz, M., Smith, T.L., Holmes, R.P., and Kummerow, F.A. (1983) Fed. Proc. 42, 1186, Abstr. No. 5236.
11. Bieri, J.G., Stoewasnd, G.S., Briggs, G.M., Phillips, R.W., Woodard, J.C., and Knapka, J.J. (1977) J. Nutr 107, 1340-1348.
12. Inkpen, C.A., Arris, R.A., and Quakenbush, F.W. (1969) J. Lipid Res. 10, 277-282.
13. Mahfouz, M., and Holman, R.T. (1980) Lipids 15, 63-65.
14. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, A.J. (1951) J. Biol. Chem. 193, 265-275.
15. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497-509.
16. De Schrijver, R., and Privett, O.S. (1982) Lipids 17, 27-34.
17. Morrison, W.R., and Smith L.M. (1964) J. Lipid Res. 5, 600-608.
18. Steel, R.G., and Torrie, J.H. (1960) in Principles and Procedures of Statistics, pp. 107-114, McGraw-Hill Book Comp., Inc., N.Y.
19. Veerkamp, J.H., and Broekhuyse, R.M. (1976) in Techniques for the Analysis of Membrane Lipids in Biochemical analysis of Membranes, (Maddy, A.H., ed.) pp. 269-270, John Wiley & Sons, Inc., New York.
20. Stewart, J.C.M. (1980) anal. Biochem. 104, 10-14.
21. Chen, R.F., and Bowman, R.L. (1965) Science 147, 729-732.
22. Catherine, T.H., and Wills, E.D. (1978) Biochem. J. 174, 585-593.
23. Brenner, R.R. (1974) Mol. Cell. Biochem. 3, 41-52.
24. Cunnane, S.C., and Wahle, K.W. (1981) Lipids 16, 771-774.
25. Actis Dato, S.M., Catala, A., and Brenner, R.R. (1973) Lipids 8, 1-6.
26. Svensson, L. (1983) Lipids 18, 171-178.
27. Jeffcoat, R., and James, A.T. (1977) Lipids 12, 469-474.
28. Ullman, D., and Sprecher, H. (1971) Biochim. Biophys. Acta 248, 61-70.
29. Mohrhauer, H., Rahm, J.J., Seufert, J., and Holman, R.T. (1971) J. Nutr. 91, 521-527.
30. Shimp, J.L., Bruckner, G., and Kinsella, J.E. (1982) J. Nutr. 112, 722-735.
31. Kurata, N., and Privett, O.S. (1980) Lipids 15, 1029-1036.
32. Thain, Q.S., Blum, M., and Carafoli, E. (1981) Eur. J. Biochem. 121, 5-13.
33. DeSchrijver, R. and Privett, O.S. (1982) J. Nutr. 112, 619-626.
34. Sklar, L.A., Milijanich, G.P., and Dratz, E.A. (1979) Biochemistry 18, 1707-1716.
35. Lipiello, P.M., Holloway, C.T., Garfield, S.A., and Holloaway, P.W. (1979) J. Biol. Chem. 254, 2004-2009.
36. Jones, P.D., Holloway, P.W., Pellutto, R.O., and Wakil, S.J. (1969) J. Biol. Chem. 244, 744-754.
37. Holloway, P.W. (1971) Biochemistry 10, 1556-1560.

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Lipid Composition and Protoplast-forming Capacity of *Streptomyces antibioticus*

M.C. ZUÑEDA, J.J. GUILLENEA, J.B. DOMINGUEZ, A. PRADO and F.M. GOÑI*,
Departments of Biology and Biochemistry, Faculty of Science, P.O. Box 644, Bilbao, Spain

ABSTRACT

The lipid and fatty acid composition of a strain of *Streptomyces antibioticus* has been studied as a function of culture age and glycine concentration in the growth medium. Under all conditions, the main polar lipids were phosphatidylethanolamine, cardiolipin and phosphomannoinositides in order of decreasing abundance; no ornithinolipids were detected. Acylglucoses and menaquinones were found among the nonpolar lipids. The main fatty acids present were anteiso 15:0 and anteiso 17:0. The lipid composition of the cells varied with the age of the culture, but no uniform pattern of variation was found in the cultures grown on different amounts of glycine. Among the cells harvested at the end of the exponential phase of growth, those grown on 2% glycine give the highest yield of protoplast formation. These cells were found to contain low amounts of nonpolar lipids and of phosphatidylethanolamine, and high proportions of anteiso fatty acids. We propose that the membrane bilayer of these cells, because of its peculiar lipid composition, is particularly stable and fluid. As a consequence, the yield and stability of derived protoplasts should be increased.
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INTRODUCTION

Cell walls from bacteria belonging to the genus *Streptomyces* are sensitive to lysozyme. Protoplast formation was first described in these microorganisms by Romano and Nickerson (1). Later the presence of glycine in the culture medium was found to considerably increase the yield of protoplasts from several *Streptomyces* species (2,3).

Although protoplast formation depends mainly on the composition of cell-wall proteoglycan and polysaccharides, the lipid composition of the mycelial membrane may possibly influence either the action of the lytic enzymes or the stability of the newly formed protoplast. In a previous study, we found data supporting such a relationship for a case of fungal protoplast formation (4).

Few systematic studies on the lipid composition of *Streptomyces* mycelia have been made. Most of our knowledge in this field has been reviewed by Batrakow and Bergelson (5). In this paper we summarize our studies on the lipid composition of *S. antibioticus* and its variation over time when grown on media containing different amounts of glycine.

MATERIALS AND METHODS

S. antibioticus, strain no. 3213, from the Colección Española de Cultivos Tipo (CECT), was used throughout this study. The culture medium was Okanishi's S medium (3) to which glycine was added, when required, in order to obtain final concentrations of 0.5%, 1.0% and 2.0% (w/v). The pH was adjusted to 7. Cells were grown in batches in 250 ml erlenmeyer flasks containing 50 ml medium. Cultures were inoculated with 5×10^7 becterial spores and incubated in a Gallenkamp orbital incubator at 200 rpm at 29 C.

Mycelia were harvested at previously fixed times by centrifugation at 1,500 x g for 25 min and washed 3 times with distilled water under the same conditions. The pellets were freeze-dried and stored at -20 C before lipid extraction.

Protoplasts were prepared as follows. Mycelia (20 mg dry weight), harvested as described, were resuspended in 10 ml medium P, according to Okanishi et al. (3) and treated with lysozyme (final concentration 0.7 mg/ml) at 37 C for 2 hr with gentle shaking. After pouring through cotton batting, the suspension was diluted with medium P and protoplasts counted in a Petroff-Hausser counting chamber.

Nonpolar lipids were extracted from 50 mg freezedried mycelia with 5 ml ice-cold acetone for 5 min at 4 C. The appropriate control experiments revealed that this treatment was highly selective and exhaustive for nonpolar lipids, and that it significantly increased the total yield of lipid extraction. The extract was separated by vacuum filtration through No. 1 Whatman paper and evaporated to a convenient volume by means of a rotatory evaporator. The mycelial residue was extracted twice with 4.75 ml of chloroform/methanol/water (2:1:0.8, v/v/v) according to Bligh and Dyer (6) for

To whom correspondence should be addressed.

polar lipid extraction. This approach was found to be superior to other extraction procedures for the material under study. The recovered extract was also evaporated to near dryness under reduced pressure.

Polar and neutral lipids were quantified microgravimetrically; their sum was considered as the "total lipid" value.

Nonpolar lipids were separated by thin layer chromatography (TLC) on Silica Gel H plates, with petroleum ether (b.p. 50-60 C)/ethylether/acetic acid (90:10:1, v/v/v) (7). The spots were visualized by means of iodine vapor and identified by their R_f values and specific reactions (8,9) as well as by comparison with authentic standards. Acylglycerides were quantified by a chromotropic acid reaction (10). Polar lipids were also separated by TLC on Silica Gel H plates, using as solvent chloroform/methanol/water (65:25:5, v/v/v) (11) and identified by procedures similar to those used for the neutral lipids. Lipid phosphorus was determined colorimetrically (12). The amount of phospholipids was estimated by multiplying the value for lipid P by 25.

Sugar-containing lipids were hydrolyzed in 1 N methanolic HCl at 60 C for 1 hr, and the resulting free monosaccharides were separated by descending paper chromatography and identified by means of standards (13). Lipid-bound sugars were determined by the Dubois phenol technique, as described by Kates (8).

Fatty acid components were transesterified to their methyl esters in the presence of boron trifluoride (14) and analyzed by gas liquid chromatography (GLC), as described previously (15). peaks were identified by comparing their retention times with those of authentic standards and quantified by a digital integrator.

Extraction and chromatographic solvents, as well as silica gel were obtained from Merck (Darmstadt, West Germany), lipid and fatty acid standards from Applied Science (Oud-Beijerland, Netherlands), gas chromatographic supplies from Xpectrix (Barcelona, Spain), bacteriological products from Difco (Detroit, MI). All other reagents were analytical grade.

RESULTS

The main nonpolar lipid constituents of *S. antibioticus* were free fatty acids, diacylglycerols, triacylglycerols, menaquinones and acylglucoses. They represented altogether, as determined by microgravimetry, 20-35% of the total lipids. All the polar lipids detected were phospholipids – phosphomannoinositides, lysophosphatidylethanolamine, phosphatidylglycerol, phosphatidylethanolamine, cardiolipin, and N-methyl-phosphatidylethanolamine.

The presence of glycine in the culture medium significantly modified the kinetics of growth of *S. antibioticus* (Table 1). Although the shape of the cell dry weight vs the time curve was always the same, increasing glycine concentrations increased the lag time, slowed down the growth rate and decreased the biomass yield. Four points were selected for our study at each glycine concentration, representing the middle of the exponential phase, the end of the exponential phase, stationary phase, and a point that could be called "aged culture." These points were determined in each case by means of absorbance and viability measurements, as well as by morphologic observations of the cells. The corresponding kinetic data together with the protoplast yields are summarized in Table 1. Protoplasts were usually prepared from mycelia harvested toward the end of the exponential phase. The yield of protoplast formation was increased ca. 4-fold when glycine concentration was increased from 1% to 2%.

Table 2 shows the lipid composition of *S. antibioticus* grown on media containing various glycine concentrations. Very small differences were found in cells grown on 0.0%, 0.5% or 1.0% glycine. The total lipid contents of the cells did not vary considerably with culture age or glycine concentration. The nonpolar-to-polar lipid ratio also remained fairly constant, between 1/2 and 1/3, except when glycine concentration in the medium was 2%. Then, very high proportions of phospholipids were found at the end of the exponential and stationary phases. Among the nonpolar lipids, diacyl and triacylglycerols were quantitated to obtain further insight into their involvement in protoplast stability. Although the proportions of these nonpolar lipid classes varied considerably by culture conditions and age, the smallest amount of diacylglycerols (1.7% of nonpolar lipids) corresponded precisely to the cells grown on 2% glycine at the end of the exponential phase, i.e., the conditions for optimal protoplast recovery.

The predominant phospholipid classes in *S. antibioticus* under almost any condition were phosphatidylethanolamine, cardiolipin and phosphomannoinositides, in order of decreasing abundance (Table 2). In general, cells grown on 0.0%, 0.5%, or 1.0% glycine behaved similarly to polar lipid composition and its variation with time; cells grown on 2% glycine, on the other hand, behaved differently. The percentage of phosphatidylethanolamine tended to be higher during the exponential growth phase, and to decrease as the cultures grew older, irrespective of the glycine concentration in the medium.

TABLE 1

The Yield of Biomass and Derived Protoplasts Obtained at Different Growth Time of *S. antibioticus* Incubated in Media Containing Various Glycine Concentrations

Growth phase	0% Glycine			0.5% Glycine			1% Glycine			2% Glycine		
	Time (hr)	Biomass[a]	Protoplast formation[b]	Time (hr)	Biomass[a]	Protoplast formation[b]	Time (hr)	Biomass[a]	Protoplast formation[b]	Time (hr)	Biomass[a]	Protoplast formation[b]
1. Mid-exponential phase	20	2.7± 0.5	–	20	2.2± 0.3	–	25	1.8± 0.4	–	70	1.7± 0.5	--
2. End of exponential phase	24	5.7± 0.6	2.5± 0.5	24	5.5± 1.0	2.8± 0.5	31	3.5± 0.5	2.0± 0.3	90	3.9± 0.5	9.6± 1.1
3. Stationary phase	31	7.1± 0.2	–	31	5.0± 0.6	–	40	4.4± 0.5	–	112	3.4± 0.3	--
4. Aged culture	40	5.6± 0.2	–	40	4.9± 0.3	–	48	4.5± 0.3	–	150	3.2± 0.4	--

[a]Biomass is expressed as mg cell dry wt/ml culture.
[b]Number of protoplast X 10^{-5} formed/mg mycelial dry wt. Figures correspond to means of 4 independent preparations ± SD.

However, the proportion of this phospholipid was markedly decreased at all stages of growth when glycine concentration was 2%, compared with the corresponding stages at lower glycine concentrations. Cardiolipin made up ca. 20% of the cell phospholipid under most growth conditions, except when the 2% glycine cultures became aged, in which case the proportion of cardiolipin was 34.3%. The proportion of phosphomannoinositides tended to increase ca. 2-fold as the cells grew older, except with 2% glycine cells, when this pattern was not observed. The remaining polar lipid classes, lysophosphatidylethanolamine, phosphatidylglycerol and N-methylphosphatidylethanolamine, were present only in very minor proportions, and did not show any particular pattern of variation in culture age or glycine concentration. Again, cells grown on 2% glycine are to be noted by their relatively high contents of lysophosphatidylethanolamine.

The amount of lipid-linked sugars in *S. antibioticus* is also shown in Table 2. The amount of sugars related to polar lipids followed as expected, the variations in phosphomannoinositide contents, although the number of sugar residues per phosphomannoinositide molecule may vary. Sugars associated with nonpolar lipids, presumably in the form of acylglucoses, were more abundant in the cells grown on 0.0% or 0.5% glycine (data not shown); however, no apparent correlation between nonpolar lipids other than di- and triacylglycerols, and nonpolar lipid-linked sugars was found.

The fatty acid distribution in nonpolar and polar lipids of *S. antibioticus* was also studied as a function of glycine concentration in the growth medium. The relevant results are summarized in Table 3. Branched-chain and saturated-linear fatty acids were found almost exclusively. The fatty acid composition of nonpolar lipids was very simple: 2 fatty acids, anteiso 15:0 and anteiso 17:0 made up more than 80% of the total; increasing the glycine concentration seemed to slightly increase the proportion of the former, at the expense of the latter. A similar behavior was found for iso 16:0 and 16:0, which together make ca. 10% of the total fatty acids. Other species were present but in negligible proportions. Polar lipids had a more complex fatty acid composition. Contrary to the situation with nonpolar lipids, palmitic acid (16:0) was present in proportions similar to those of anteiso 15:0 and anteiso 17:0 in phospholipids. Other linear saturated fatty acids, such as 14:0 and 17:0, were also more abundant than in nonpolar lipids. Finally, the monounsaturated oleic acid

TABLE 2

Lipid Composition of *S. antibioticus* Grown on Media Containing 1 or 2% Glycine: Variations with Culture Phase

	1% Glycine				2% Glycine			
Growth phase[a]	1	2	3	4	1	2	3	4
Total lipids (% dry wt)	2.0 ± 0.2	2.1 ± 0.1	2.6 ± 0.4	2.8 ± 0.3	2.3 ± 0.2	2.5 ± 0.5	2.6 ± 0.3	2.8 ± 0.3
% Total lipids								
Nonpolar	25.3 ± 3.3	28.6 ± 2.8	22.6 ± 2.5	26.4 ± 3.5	29.3 ± 3.0	5.4 ± 1.8	11.1 ± 2.5	35.3 ± 3.0
Polar	74.7 ± 6.7	71.4 ± 7.3	77.4 ± 7.5	73.8 ± 6.5	70.7 ± 7.0	94.6 ± 6.6	88.9 ± 6.6	64.7 ± 7.0
% Nonpolar lipids[b]								
DG	28.6 ± 1.5	29.5 ± 2.0	14.3 ± 0.6	17.8 ± 0.7	7.6 ± 2.9	1.7 ± 0.5	3.0 ± 0.9	15.2 ± 0.9
TG	11.1 ± 1.9	13.7 ± 1.6	27.6 ± 0.9	30.8 ± 2.1	18.4 ± 1.1	30.0 ± 1.0	24.3 ± 1.3	27.8 ± 1.0
% Polar lipids[c]								
PMI	8.4 ± 0.4	10.0 ± 0.4	9.0 ± 0.8	12.6 ± 0.9	19.0 ± 0.5	17.1 ± 1.0	22.4 ± 1.0	16.0 ± 1.1
LPE	1.2 ± 0.1	0.8 ± 0.1	2.5 ± 0.2	4.4 ± 0.2	8.0 ± 0.3	4.8 ± 0.1	7.0 ± 0.3	10.7 ± 0.6
PG	4.0 ± 0.1	1.0 ± 0.1	1.8 ± 0.1	4.5 ± 0.1	4.0 ± 0.0	4.1 ± 0.1	4.6 ± 0.2	8.3 ± 0.7
PE	69.2 ± 7.0	70.9 ± 7.2	66.0 ± 6.7	57.6 ± 6.1	43.4 ± 6.7	53.4 ± 7.1	39.6 ± 6.7	9.2 ± 5.3
CL	16.5 ± 2.0	15.9 ± 2.2	20.3 ± 1.9	20.7 ± 2.2	24.9 ± 1.7	19.1 ± 1.6	20.3 ± 1.5	34.3 ± 2.0
NMPE	0.5 ± 0.2	–	–	0.4 ± 0.1	1.3 ± 0.2	2.4 ± 0.0	1.8 ± 0.3	2.0 ± 0.3
Lipid-linked sugars[d]								
Nonpolar	270 ± 20	109 ± 47	142 ± 33	121 ± 18	135 ± 16	156 ± 19	115 ± 32	121 ± 34
Polar	165 ± 39	181 ± 22	235 ± 63	411 ± 58	235 ± 15	160 ± 14	196 ± 15	282 ± 38

Figures correspond to mean values of cells from three different batches ± SD.

[a]1: Mid-exponential phase; 2: end of exponential phase; 3: stationary phase; 4: aged culture.

[b]DG: diacylglycerols; TG: triacylglycerols.

[c]PMI: phosphomannoinositides; LPE; lysophosphatidylethanolamine; PG: phosphatidylglycerol; PE: phosphatidylethanolamine; CL: cardiolipin; NMPE: N-methyl phosphatidylethanolamine.

[d]μg Sugar/100 mg dry wt lipid.

TABLE 3

Precent Distribution of the Main Fatty Acids Associated with the Neutral Lipids and Phospholipids of *S. antibioticus* Grown on Media Containing Various Amounts of Glycine[a]

Fatty acid	Relative retention time	Nonpolar lipids				Polar lipids			
		0% Gly	0.5% Gly	1% Gly	2% Gly	0% Gly	0.5% Gly	1% Gly	2% Gly
Iso 14:0	0.51	0.5	0.8	1.4	1.8	3.2	–	–	3.3
14:0	0.58	0.7	0.5	0.5	0.5	5.2	4.7	8.6	7.2
Anteiso 15:0	0.70	42.6	44.0	47.3	47.0	27.9	21.1	17.4	26.1
Iso 16:0	0.88	3.7	8.3	10.8	7.5	6.9	1.7	7.1	7.0
16:0	1.00	7.6	6.8	4.8	2.6	19.4	29.3	27.9	19.4
Anteiso 17:0	1.21	43.4	38.1	34.9	39.4	28.2	25.4	18.2	25.8
17:0	1.78	0.9	0.9	–	0.5	3.5	6.7	4.2	3.6
18:0	1.84	–	–	0.5	1.9	–	–	–	–
18:1	2.03	0.9	0.4	–	–	6.3	11.2	6.4	4.1
Unidentified		–	–	–	–	–	–	10.2	3.7
Total anteiso		86.0	82.1	82.2	86.4	56.1	46.5	35.7	51.9
Iso/anteiso ratio		0.05	0.11	0.15	0.10	0.18	0.04	0.20	0.20

Figures correspond to mean values of 2 independent experiments.
[a]Mycelia were harvested at the beginning of the stationary phase.

(18:1), as well as an unidentified species, appeared in small but significant proportions in the polar lipid fraction. In spite of various changes in fatty acid composition as glycine concentration is increased, polar lipids of cells grown on 2% glycine were found to return to virtually the same fatty acid distribution of those grown on a glycine-free medium.

DISCUSSION

In spite of their obvious economic importance as producers of antibiotics, bacteria belonging to the genus *Streptomyces* have not been thoroughly explored from the point of view of lipid composition. The present study constitutes, to the authors' knowledge, the first attempt to determine, in some detail, the lipid composition of *Streptomyces* species and their variation with culture growth, and yet the economic importance of *S. antibioticus* makes it a potential material for genetic engineering studies. Bacterial protoplasts are often required in fusion experiments related to genetic studies. In this paper, we report that the protoplast formation yield of *S. antibioticus* is much higher when glycine is present in the medium at a 2% (w/v) concentration, which suggests that under these conditions protoplasts formed are more stable. The present work also includes the changes in lipid composition of *S. antibioticus* grown on various glycine concentrations, and shows significant variations when the concentration of this amino acid is raised to 2% (w/v).

Our data on lipid composition generally accord with previous studies of different *Streptomyces* species (5,16-20). The nonpolar lipid classes included menaquinones and acylglucoses, confirming the findings of Batrakow and Bergelson (5).

All the polar lipids found are of the phosphoglyceride type; no ornithinolipid of the sort described by other authors (5) in *Streptomyces* was detected in our case. The main phospholipid classes found were phosphatidylethanolamine, cardiolipin and phosphomannoinositides; this is in accord with the observations of Kataoka and Nojima (16) in *Streptomyces griseus*, although the proportions are slightly varied, with a higher phosphatidylethanolamine proportion in our case. In *Streptomyes sioyaensis*, phosphatidylethanolamines of different fatty acid composition can be resolved into 2 chromatographic spots by TLC (17), but this not the case in *S. antibioticus*. From the quantitative point of view, the amount of phospholipids in our preparations varied according to culture conditions and age, from 1.5% to 2.4% cell dry wt, which is less than the 3.1% found in *S. griseus* (16).

The fatty acid distributions of polar and nonpolar lipids of *S. antibioticus* also agree with data from other *Streptomyces* species (5,21-23). The relatively high proportion of anteiso fatty acids is worth noting. In our case, anteiso 15:0 and anteiso 17:0 make up the bulk of the fatty acids (Table 3). No anteiso chains were found in *S. griseus* (16), while in other cases (2) only anteiso 15:0 was present in significant amounts. Ballio and Barcellona (23), examining 13 *Streptomyces* strains, only found significant amounts of anteiso 17:0 (24.5%) in one of them, namely *Streptomyces sp.* P.R.L. In their study, the iso/anteiso ratio varied from

0.5 in *S. venezuelae* to 3.6 in *S. gelaticus*; whereas, in our case, it was never higher than 0.2. Our results, on the other hand, are very similar to those found in some *Streptomyces* glycolipids (5). Apparently, the anteiso branched chains tend to fluidize the membranes; they do so less effectively than the *cis*-unsaturated fatty acids, but more than their iso homologues (24). Thus, the membranes of *S. antibioticus* would be more fluid than those of other *Streptomyces* species studied up to now.

Lipid composition varies with culture age in a complex way, depending on the amount of glycine in the medium. A few clear trends may be seen in Table 2. These trends usually occur in the cultures containing 0.0%, 0.5% and 1.0% glycine, while the situation changes for 2.0% glycine. In summary, when glycine is present in the medium at 2% concentration; (a) the amount of total lipid does not vary; (b) the proportion of polar lipids is highest; (c) among nonpolar lipids, the proportion of diacylglycerols is remarkably low; (d) the proportion of phosphatidylethanolamine decreases, accompanied by an increase in phosphomannoinositides, and (e) the fatty acid distribution of the polar lipids is virtually identical to that of cells grown on a glycine-free medium. Most of the differences mentioned here are especially remarkable at the end of exponential phase (growth phase 2), i.e., at the point when cells are usually harvested for protoplast preparation. These results may be rationalized in view of the present knowledge of the physical chemistry of phospholipids as follows. Although most nonpolar lipids are present in the form of cytoplasmic vacuoles, some are incorporated into the cell membranes, decreasing the stability of the bilayer (25); at 2% glycine, the proportion of nonpolar lipids at the end of the exponential phase is remarkably low. In addition, phosphatidylethanolamine is known for its tendency to give rise to H_{II} hexagonal (nonbilayer) structures (25); the point of lowest phosphatidylethanolamine proportion at growth phase 2 corresponded precisely to the condition of highest protoplast yield. Finally, when the glycine concentration is 2% (w/v), the proportion of anteiso fatty acids in phospholipids is relatively high (Table 3), making the membranes more fluid at a given temperature (24); we have observed in a different system (4) that membrane fluidity is an important factor governing protoplast stability. We can thus conclude that when glycine is present in the growth medium at a 2% (w/v) concentration, the protoplasts obtained from *S. antibioticus* are more stable, and consequently protoplast yield is higher, because under these conditions the membrane lipid bilayer is more stable and more fluid.

REFERENCES

1. Romano, A.H., and Nickerson, W.J. (1956) J. Bacteriol., 72, 478-482.
2. Sagara, Y., Fukui, K., Ota, F., Yoshida, N., Kashiyama, T., and Fujimoto, N. (1971) Jpn. J. Microbiol. 15, 73-84.
3. Okanishi, M., Suzuki, K., and Umezawa, H. (1974) J. Gen. Microbiol. 80, 389-400.
4. Domínguez, J.B., Goñi, F.M., and Uruburu, F. (1978) J. Gen. Microbiol. 108,111-118.
5. Batrakow, S.G., and Bergelson, L. (1978) Chem. Phys. Lipids. 21, 1-29.
6. Bligh, E.G., and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911-917.
7. Mangold, H.K., and Malins, D.C. (1960) J. Am. Oil Chem. Soc. 37, 383-385.
8. Kates, M. (1972) Techniques of Lipidology, North-Holland, Amsterdam.
9. Goñi, F.M., and Macarulla, J.M. (1977) Laboratorio 64, 501-520.
10. Van Handel, E., and Zilversmit, D.B. (1957) Lab. Clin. Med. 50, 152-158.
11. Smith, I., and Seakins, J.W.T. (1976) Chromatographic and Electrophoretic Techniques, Midland Books Ltd., London.
12. Bartlett, G.R. (1959) J. Biol. Chem. 234, 446-468.
13. Goñi, F.M., Gómez, J.C., and Santiago, E. (1978) Rev. Esp. Fisiol. 33, 331-336.
14. Morrison, W.R., and Smith L.M. (1964) J. Lipid Res. 5:660-663.
15. Regúlez, P., Pontón, J., Domínguez, J.B., Goñi, F.M., and Uruburu, F. (1980) Can J. Microbiol. 26, 1428-1437.
16. Takaoka, T., and Nojima, S. (1967) Biochim. Biophys Acta 144, 681-695.
17. Kawanami, J., Kimura, A., Nakagawa, Y., and Otauka, H. (1969) Chem. Phys. Lipids 3, 69-75.
18. Kawanami, J. (1971) Chem. Phys. Lipids 7, 159-168.
19. Kawanami, J., and Otsuka, H. (1969) Chem. Phys. Lipids 3, 135-142.
20. Kawanami, J., Kimura, A., and Otsuka, H. (1968) Biochim. Biophys. Acta 152, 808-816.
21. Ballio, A., Barcellona, S., and Boniforti, L. (1965) Biochem. J. 94, 11c-13c.
22. Ballio, A., Barcellona, S., and Salvatori, T. (1968) J. Chromatogr. 35, 211-215.
23. Ballio, A., and Barcellona, S. (1968) An. Inst. Pasteur 114, 121-132.
24. Silvius, J.R., and McElhaney, R.N. (1980) Chem. Phys. Lipids 26, 67-76.
25. Chapman, D. (1968) Biological Membranes: Physical Fact and Function, Academic Press, London.

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COMMUNICATIONS

Steroids from Aquatic Organisms: V. Main Sterols of the Fresh Water Snail, *Biomphalaria tenagophila*

ALICIA M. SELDES and EDUARDO G. GROS*, *Departamento de Quimica Organica and UMYMFOR, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Pab. 2, Ciudad Universitaria, 1428 Buenos Aires, Argentina*

ABSTRACT

The free sterols of the snail, *Biomphalaria tenagophila,* were isolated and characterized by means of gas chromatography and gas chromatography-mass spectrometry techniques. The mixture contains the common steroids found in freshwater molluscs, principally cholesterol. The presence of cholesta-5,7-dien-3β- ol is without precedent in freshwater organisms. The occurrence of 7-oxo-cholesterol in freshwater snails, but not bivalves, from the same freshwater source, would indicate a difference in the physiology of such snails and bivalves.
Lipids 19:229-230, 1984.

Chemotherapy is one of the most valuable methods in the fight against schistosomiasis. A knowledge of the chemical composition of the schistosomiasis-transmitting snail, *Biomphalaria tenagophila,* would be useful in determining the specific conditions that the snail vector has to fulfill to play host to the schistosoma. Accordingly, and in connection with our previous studies on sterols from aquatic organisms (1-4), we report here the composition of the sterols of this mollusc.

MATERIALS AND METHODS

Biomphalaria tenagophila specimens were collected at the Parana River and frozen immediately. The soft tissue from the molluscs was extracted as previously described (3). The extracts were kept under nitrogen at -15 C until they were processed. The extracts were chromatographed on a silica gel column with elution by toluene-ethyl acetate mixtures of increasing polarity. The crude sterol mixture was recrystallized from ethanol to a clean product that gave only one spot on thin layer chromatography (TLC) on silica gel G plates using petroleum ether/ethyl acetate (8:2, v/v) as developing solvent.

The mixture was analyzed by gas chromatography (GC) using a Hewlett-Packard 5840 instrument equipped with a FID detector and a 12 m × 0.2 mm fused capillary column coated with methyl silicone (Hewlett-Packard). The steroids were chromatographed between 200 and 290 C at a rate of 8°/min using helium as the carrier gas. Quantitation was by automatic integration of the peaks using stigmasterol as the internal standard. Relative detector responses were determined using an artificial mixture of the most common sterols (C_{28} and C_{29}). The identity of the steroids was assigned by gas chromatography-mass spectrometry (GC-MS) using a Varian MAT CH7-A MS coupled to a Varian 1440 GC and interfaced to a Varian MAT Data System 166 computer. Analyses of the trimethylsilyl ether derivatives (5) (hexamethyldisilazane/trimethylchlorosilane/pyridine, 3:3:10, v/v/v) were performed under the same conditions.

*To whom correspondence should be addressed.

RESULTS AND DISCUSSION

Analyses of the free sterols by GC and GC-MS as indicated above are recorded in Table 1, which shows the respective structures and relative proportions. As expected, the sterol mixture contains a large proportion of cholesterol (6,7). The remaining sterols are common in freshwater molluscs; some of them may be dietary in origin since the snails feed on plants. As far as we know, the presence of $\Delta^{5,7}$ sterols has never been reported in freshwater molluscs that contain mainly Δ^5 sterols.

An uncommon steroid with a retention time higher than that of sitosterol is present in these molluscs. This steroid presents, as a free sterol, a M^+ at m/z 400 and fragments at 385 (M - 15), 382 (M - 18), 367 (M - 33), 287 (M - 113), 269 (M - 113 - 18), 245 (M - 113 - 42), 213 (M - 113 - 42 - 18), indicating a C_8H_{17} side chain and two oxygenated functions on the tetracyclic system. The MS of the TMS derivative presents the corresponding fragments, as well as ions at m/z 135 and 129, but lacks a M - 129 fragment. These spectral properties are identical to those from synthetic 7-oxo-cholest-5-en-3β-ol (8) that was analyzed under the same conditions. This compound has been found in several marine bivalves (9), but to the best of our knowledge has never been reported in freshwater organisms. Note that we have found the same oxosterol (identical RRT and MS) in other freshwater snails, *Ampullaria canaliculata* and *Ampullaria insularum,* and in a marine gastropod, *Patinigera magallanica* (Romero and Seldes, unpublished

TABLE 1

Sterol Composition of the Snail, *Biomphalaria tenagophila*

Sterols	MS Characteristic Fragments	RRT[a]	Estimated percentage
22-*trans*-Cholesta-5,22-dien-3β-ol	384(M^+),366,351,300,273,271, 255,213,111,69,55	0.96	5.4
Cholest -5-en-3β-ol	386(M^+),371,368,301,275,273, 255,231,213,145,43	1.00	65.4
22-*trans*-24-Methylcholesta-5,22-dien-3β-ol	398(M^+),380,365,300,271,255, 213,69,55	1.02	2.7
Cholesta-5,7-dien-3β-ol	384(M^+),366,351,271,253,211, 43	1.03	5.3
24-Methylcholest-5-en-3β-ol	400(M^+),385,382,367,315,289, 273,213,105,43	1.07	8.1
22-*trans*-24-Ethylcholesta-5,22-dien-3β-ol	412(M^+),397,394,379,351,300, 273,271,255,69,55	1.10	5.7
24-Ethylcholest-5-en-3β-ol	414(M^+),396,381,368,329,303, 255,213,91,55	1.15	6.3
7-Oxo-cholest-5-en-3β-ol	400(M^+),385,382,367,287,269, 245,213,197,192,161,43	1.21	1.2

[a]Relative retention times (RRT) of the free sterols to cholest-5-en-3β-ol.

results).

The results summarized in Table 1, which agree with previous results, are evidence that the sterol composition of the freshwater molluscs examined is much simpler than that of its marine counterpart. The suggestion has been made (6) that this simpler sterol composition is a result of the less stringent requirements of the membrane functions in animals living in low-salinity water.

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REFERENCES

1. Romero, M.S., and Seldes, A.M. (1983) Experientia 39, 258-259.
2. Romero, M.S., and Seldes, A.M. (1983) J. Natl. Prod. (Lloydia) 46, 588-590.
3. Seldes, A.M., and Gros, E.G. (1983) Rev. Latinoamer. Quim. 14, 33-35.
4. Rovirosa, J., Muñoz, O., San Martín, A., Seldes, A.M., and Gros, E.G. (1983) Lipids 18, 570-572.
5. Brooks, C.J.W., Horning, E.C., and Young, J.S. (1968) Lipids 3, 391-402.
6. Popov, S., Stoilov, I., Marekov, N., Kovachev, G., and Andreev, St. (1981) Lipids 16, 663-669.
7. Pollero, R.J., Gros, E.G., and Brenner, R.R. (1983) Lipids 18, 100-102.
8. Aringer, L., and Nordström, L. (1981) Biomed. Mass Spectrom. 8, 183-203.
9. Pastoriza, L., and Rodriguez, B. (1982) Invest. Pesq. 46, 83-89; C. A. 69623a (1982).

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Response of *Drosophila* to *cis*- and *trans*-22-Dehydrocholesterol II. Ability of Sterols other than Cholesterol to Overcome the Toxicity of the *trans* Isomer[1]

HENRY W. KIRCHER, FUMIKO U. ROSENSTEIN and JAMES C. FOGLEMAN,[2] *
Department of Nutrition and Food Science, University of Arizona, Tucson, AZ 85721

ABSTRACT

Four species of *Drosophila* were tested, for two generations, for viability on media containing 13 different C_{27}, C_{28} and C_{29} sterols with and without *trans*-22-dehydrocholesterol. Only cholesterol was effective for overcoming the toxic effect of the *trans* derivative for all 4 species. The species varied in their ability to use the 13 sterols for growth, maturation and reproduction for 2 generations. The most tolerant species. *D. pseudoobscura,* could use 11 of the 13 sterols for structural and hormonal functions, whereas the most sensitive species, *D. mettleri,* could survive adequately on only 4 and less than adequately on another 3 of the 13 compounds. Desmosterol could not be used by any species, even for 1 generation.
Lipids 19:231-234, 1984.

INTRODUCTION

In the preceding paper (1), we demonstrated that the addition of *trans*- but not *cis*-22-dehydrocholesterol to media prevented larval maturation of several species of *Drosophila* and that the concomitant addition of cholesterol prevented this toxic effect. In addition, we showed that the presence of alkyl groups at C_{24} in other *trans*-Δ^{22} sterols (brassicasterol, stigmasterol, 5,6-dihydroergosterol, spinasterol) eliminated the larval mortality observed with the unsubstituted side chain of *trans*-22-dehydrocholesterol (*trans*-DHC). In this paper, we present the results obtained by incorporating numerous C_{27}, C_{28} and C_{29} sterols into *Drosophila* medium with and without added *trans*-DHC to further investigate its toxic effect (Table 1). Four species of *Drosophila* were chosen for these studies on the basis of their previous responses to dietary sterols (1). *Drosophila pseudoobscura* was the most tolerant, followed by *D. melanogaster* and *D. mojavensis,* and finally by *D. mettleri,* the species most sensitive to variations in the structure of sterols added to the medium.

MATERIALS AND METHODS

All 4 species of *Drosophila* were obtained from the laboratory of W. B. Heed, Department of Ecology and Evolutionary Biology, University of Arizona, Tucson, AZ. The following sterols were used in this study in addition to those used in the previous work (1): cholestanol (2), *trans*-22-cholestenol, hydrolyzed from the acetate (3) MP 122-122.5 (vac, corr), dihydrobrassicasterol (4), sitosterol (5), 7-dehydrostigmasterol (6), and *trans*-22-dehydrodesmosterol (7) were available from earlier work. Ergosterol was a commercial sample recrystallized from alcohol; MP 165-165.5 (vac, corr) and desmosterol, MP 121.5-122.5 (vac, corr) −38.5 (C_3, $CHCl_3$) were prepared by the isomerization of 25-dehydrocholesteryl acetate with I_2 in benzene (Kircher and Rosenstein, unpublished).

All sterols were added to the axenic sterol deficient medium (1) at a concentration of 230 mg/l. Tests were initiated with NaOCl-sterilized *Drosophila* eggs, as described previously (1). F_1 adults emerging from each of the 5 replicate bottles/test were axenically transferred to fresh bottles of the same medium they had emerged from. When F_2 larvae were visible, the F_1 population was removed. Results are expressed by scoring the egg to F_1 and F_2 adult viability for each sterol supplement.

RESULTS AND DISCUSSION

The ability of the 4 species of *Drosophila* to use the various test sterols for growth, maturation and reproduction under axenic conditions is shown in Table 2. All species were able to live for 2 generations on the 3 C_{28} (ergostane) and C_{29} (stigmastane) derivatives and cholesterol. Overall the best performance was on the 3 stigmastanes and this suggests that the widely occurring sitosterol and stigmasterol are the

[1] Arizona Agricultural Experiment Station journal article no. 3811.
[2] Present address: Department of Biological Sciences, University of Denver, Denver, CO 80208.
*To whom correspondence should be addressed.

TABLE 1

Nomenclature

Cholestanol	5α-Cholestan-3β-ol
Cholesterol	Cholest-5-en-3β-ol
Lathosterol	5α-Cholest-7-en-3β-ol
trans-DHC	Cholesta-5-22E-dien-3β-ol
cis-DHC	Cholesta-5-22Z-dien-3β-ol
t-22-Cholesterol	5α-Cholest-22E-en-3β-ol
Desmosterol	Cholesta-5,24-dien-3β-ol
t-22-Dehydrodesmosterol	Cholesta-5,22E,24-trien-3β-ol
Dihydrobrassicasterol	Ergost-5-en-3β-ol
Brassicasterol	Ergosta-5,22E-dien-3β-ol
Ergosterol	Ergosta-5,7,22E-triene-3β-ol
Sitosterol	Stigmast-5-en-3β-ol
Stigmasterol	Stigmasta-5,22E-dien-3β-ol
7-Dehydrostigmasterol	Stigmasta-5,7,22E-trien-3β-ol

TABLE 2

Ability of 4 Species of *Drosophila* to Use Various Dietary Sterols for Growth, Maturation and Reproduction (Eggs → F_1 Adults → F_2 Adults)[a,b]

Sterols	Double bonds	*D. pseudoobscura*		*D. melanogaster*		*D. mojavensis*		*D. mettleri*	
		F_1	F_2	F_1	F_2	F_1	F_2	F_1	F_2
C_{27}									
Cholestanol	none	+	+	+	0	+	0	+	0
Cholesterol[c]	5	+	+	+	+	+	+	+	+/0
Lathosterol	7	+	+	+	+	+	0	+	0
t-22-Cholestenol	22E	+	+	+	W	+	+/0	not tested	
trans-DHC[c]	5,22E	0	–	0	–	0	–	0	–
cis-DHC[c]	5,22Z	+	+	+	+	+	0	W	0
Desmosterol	5,24	0	–	0	–	0	–	0	–
t-22-Dehydrodesmosterol	5,22E,24	+	+	+	+	+	0	0	–
C_{28}									
Dihydrobrassicasterol	5	+	+	+	+	+	+	+/0	+/0
Brassicasterol	5,22E	+/0	+	+	+	+	+	+/0	+
Ergosterol	5,7,22E	+	+	+	+	+	+	+/0	+/0
C_{29}									
Sitosterol	5	+	+	+	+	+	+	+	+
Stigmasterol	5,22E	+	+	+	+	+	+	+	+
7-Dehydrostigmasterol	5,7,22E	+	+	+	+	+	+	+	+

[a] + = more than 10 adults/bottle, W = 1 to 10 adults/bottle, 0 = no adults observed, +/0 = inconsistent results, some bottles produced adults, some did not.

[b] In no cases did F_1 adults produce F_2 larvae that failed to mature. All F_2 negatives (0) are caused by no eggs or eggs that failed to hatch.

[c] Data from ref. 1.

preferred dietary sterols for these saprophytic insects. The poorer viability of the most sensitive species, *D. mettleri,* on the ergostane triad indicates that, for this species, the sterols of its host plants, decaying saguaro and cardón cacti (8,9), are used by the insects, rather than the sterols of the yeasts that provide the principal nutrients for the larvae.

Other than cholesterol, none of the C_{27} (cholestane) derivatives provided for egg to F_2 viability for all of the species. Neither *trans*-DHC nor desmosterol allowed even first generation flies to develop. Cholestanol is a sparing sterol (2) and allowed maturation of F_1 flies because of the transmission of necessary sterols through the egg. The ability of *D. pseudoobscura* to go for 2 generations on cholestanol attests to its very low requirement for unsaturated sterols. The traces of sterols still evident

in the sterol deficient medium, ca. 0.01% of the dry ingredients (1), are still enough for this species to mature. The relative sensitivities of the 4 species to variations in sterol structure noted in (1) was substantiated here with 3 other compounds. Two generations of *D. pseudoobscura* and *D. melanogaster* can be reared with lathosterol and 22-dehydrodesmosterol in the medium, whereas only 1 or no generations of the other 2 species could be reared on these sterols and only *D. pseudoobscura* could use *trans*-22-cholestenol well.

Two other comparisons are of interest. In these instances, toxicity means that the insects are unable to mature to adults when a particular sterol has been added to the medium. This result can be attributed to a blocking of a necessary metabolic step by these sterols, their inability to be metabolized to an intermediate in ecdysone biosynthesis (10) or their inability to be used as a sparing sterol. The toxicity of *trans*-22-DHC is eliminated when the Δ^5 double bond is removed from the molecule, as with *trans*-22-cholestenol, and the toxicity of desmosterol is reduced when a *trans*-Δ^{22}-double bond is introduced, as with *trans*-22-dehydrodesmosterol (Table 2).

Both desmosterol and *trans*-22-dehydrodesmosterol are normal metabolites during the dealkylation of phytosterols, e.g., sitosterol and stigmasterol, by phytophagous insects (11). The inability of 4 species of *Drosophila* to utilize desmosterol and 2 species to utilize 22-dehydrodesmosterol is evidence that these insects do not dealkylate phytosterols (12).

The ability of the sterols, listed in Table 2, to overcome the toxicity of *trans*-DHC, is shown in Table 3. Cholesterol was the only sterol to allow egg to F_2 adult development for all 4 species and the only one that allowed *D. mojavensis* and *D. mettleri* to go for 2 generations when *trans*-DHC was also present in the medium. *D. melanogaster* was able to overcome the effect of *trans*-DHC with 7-dehydrostigmasterol as well and *D. pseudoobscura* could also use lathosterol, dihydrobrassicasterol and stigmasterol.

Whatever toxic effects of 0.25% *trans*-DHC has on *Drosophila* (1), they are best overcome with 0.25% cholesterol. A double bond at Δ^7 instead of Δ^5 (lathosterol) is less effective for this purpose, and a sterol with no double bonds (cholestanol) is completely ineffective. In the ergostane series, the Δ^5 derivative is best and in the stigmastane series, the $\Delta^{5,7,22}$ derivative was able to overcome *trans*-DHC toxicity most effectively. Since all of the ergostane and stigmastane derivatives could be used by all 4 species of *Drosophila* (Table 2), the toxicity of *trans*-DHC must lie in its disruption of some step in the insects' metabolism of sterols. This disruption may be the introduction of a Δ^7 bond in the nucleus or a hydroxyl group in the sterol side chain. The ability of cholesterol to overcome this toxicity better than any other sterol may be related to the greater structural similarity of cholesterol to *trans*-DHC than that of any of the other sterols tested.

TABLE 3

Ability of Various Sterols to Overcome the Toxicity of *trans*-DHC Toward 4 Species of *Drosophila* for 2 Generations[a,b,c,d]

trans-DHC plus	Double bonds	*D. pseudoobscura*		*D. melanogaster*		*D. mojavensis*		*D. mettleri*	
		F_1	F_2	F_1	F_2	F_1	F_2	F_1	F_2
Cholestanol	none	0	–	0	–	0	–	0	–
Cholesterol[c]	5	+	+	+	+	+	+	+	+
Lathosterol	7	+	+	0	–	W	0	0	–
cis-DHC[c]	5,22Z	0	–	0	–	0	–	0	–
t-22-Dehydrodesmosterol	5,22E,24	W	0	0	–	W	0	0	–
Dihydrobrassicasterol	5	+	+	W	W/0	+	0	0	–
Brassicasterol	5,22E	+/0	0	0	–	0	–	0	–
Ergosterol	5,7,22E	W	0	0	–	0	–	0	–
Sitosterol	5	0	–	0	–	0	–	0	–
Stigmasterol	5,22E	+	+/0	W	0	W	0	0	–
7-Dehydrostigmasterol	5,7,22E	+	+	+	+	W	0	0	–

[a]+ = more than 10 adults/bottle, W = 1 to 10 adults/bottle, 0 = no adults observed, +/0 = inconsistent results, some bottles produced adults, some did not.

[b]In no cases did F_1 adults produce F_2 larvae that failed to mature. All F_2 negatives (0) are caused by no eggs or eggs that failed to hatch.

[c]Data from ref. 1.

[d]230 mg *trans*-DHC plus 230 mg of various sterols/l medium (each sterol 0.25% of the weight of the dry ingredients).

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REFERENCES

1. Kircher, H.W., Phariss, R.L., Rosenstein, F.U., Baldwin, D., and Fogleman, J.C. (1982) Lipids 17, 209-214.
2. Kircher, H.W., and Gray, M.A. (1978) J. Insect Physiol. 24, 555-559.
3. Rosenstein, F.U., Caruso, R., and Kircher, H.W. (1977) Lipids 12, 297-300.
4. Kircher, H.W., and Rosenstein, F.U. (1973) Lipids 8, 453-458.
5. Kircher, H.W., and Rosenstein, F.U. (1973) Lipids 8, 97-100.
6. Kircher, H.W., and Rosenstein, F.U. (1973) J. Org. Chem. 38, 2259-2260.
7. Kircher, H.W., and Rosenstein, F.U. (1982) J. Org. Chem. 47, 1722-1724.
8. Kircher, H.W. (1982) in Ecological Genetics and Evolution (Barker, J.S.F., and Starmer, W.T., eds.) Chap. 10, pp. 144-5, Academic Press, Sydney.
9. Fogleman, J.C., Hackbarth, K., and Heed, W.B. (1981) Am. Nat. 118, 541-548.
10. Kircher, H.W. (1982) in Cholesterol Systems in Insects and Animals (Dupont, J., ed.) Chap. 1, CRC Press, Boca Raton, FL.
11. Svoboda, J.A., Kaplanis, J.N., Robbins, W.E., and Thompson, M.J. (1975) Ann. Rev. Entomol. 20, 205-220.
12. Kircher, H.W., Rosenstein, F.U., and Fogleman, J.C. (1984) Lipids 19, 235-238.

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Selective Uptake and Lack of Dealkylation of Phytosterols by Cactophilic Species of *Drosophila* [1]

HENRY W. KIRCHER, FUMIKO U. ROSENSTEIN and **JAMES C. FOGLEMAN,**[2] *
Department of Nutrition and Food Science, University of Arizona, Tucson, AZ 85721

ABSTRACT

The selective absorption of cholesterol and campesterol and discrimination against sitosterol was demonstrated with *Drosophila mojavensis* and *Drosophila nigrospiracula*, using a synthetic medium and 2 natural host cacti. Preferential absorption of trace amounts of phytosterols from a large quanity of other lipids occurred when *D. arizonensis* and *D. mojavensis* were reared from agria cactus. The absence of dealkylation of sitosterol to cholesterol was demonstrated with *D. mojavensis* and *D. mettleri*, reared on a sterol-deficient medium supplemented with sitosterol.
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INTRODUCTION

Houseflies, cockroaches and Khapra beetles, but not milkweed bugs, selectively absorb cholesterol and campesterol, in preference to sitosterol, from their diets (1-4). A much larger number of other phytophagous and omnivorous insects were shown to remove methyl and ethyl groups from C_{24} in the side chains of plant and fungal sterols by a selective dealkylation process and reduce the Δ^{24} or $\Delta^{22,24}$ intermediates of this process to cholesterol (5-10). Only 6 insects have been shown to be unable to dealkylate phytosterols so far; these are the hide beetle, house fly, milkweed bug, honey bee, Khapra beetle and leafcutter ant (5,11).

Most of the studies on the use and metabolism of sterols by species of *Drosophila* did not address these questions (12-17). In one case, however, on the basis of feeding studies and thin layer chromatography (TLC) of pupal sterols on silica gel, the researchers stated that *D. melanogaster* is able to dealkylate phytosterols and reduce Δ^7 and Δ^{22} double bonds to cholesterol (18). In more recent work, *D. melanogaster* and 3 other species were unable to mature to adults when fed a yeast mutant that lacked a $\Delta^8 \rightarrow \Delta^7$ isomerase or one that lacked a C_{24}-methyl transferase (19). In the latter case, 3 Δ^{24}-sterols accumulated in the yeast instead of ergosterol and the authors speculated that *Drosophila* may not be able to saturate the Δ^{24} double bond in the sterol side chain.

We recently noted that desmosterol (24-dehydrocholesterol) could not be used as a dietary sterol by any of the 4 species of cactophilic *Drosophila* that were tested and that one of them, *D. mettleri*, grew better on a medium that contained sitosterol than on those containing either of several cholestane or ergostane derivatives (17). This suggested that these species of *Drosophila* may be unable to dealkylate phytosterols because desmosterol is an intermediate in the phytosterol to cholesterol transformation (20). These conflicting results (phytosterol to cholesterol metabolism [18], no Δ^{24} saturase present in *Drosophila* [17,19]) induced us to determine whether or not several cactophilic species of *Drosophila*, which breed in plants of quite different chemical composition, are able to dealkylate ingested phytosterols to cholesterol. In this paper we describe the selective uptake and metabolism of sterols from synthetic media and from natural substances by several species of cactophilic *Drosophila*.

MATERIALS AND METHODS

Sources of *Drosophila* species, sterols and methods of axenic culture were as previously described (14-17). The adults emerging from axenic media were collected and kept frozen until analysis.

Flies were also reared in the laboratory on their natural substrates (21). Several large pieces of cactus were placed in 20 l cloth-covered jars and inoculated with juice from naturally decaying plants. After a week, adults of the appropriate species of *Drosophila* were put into the jars to oviposit. The F_1 and F_2 adults were collected from the jars by aspiration as they emerged, placed on wet paper for 1-2 days to evacuate their guts and kept frozen until analyzed. The flies were analyzed for sterols by conventional methods: $CHCl_3$-MeOH extraction, lipid saponification, precipitation of the sterols from the nonsaponifiable fraction

[1] Arizona Agricultural Experiment Station journal article no. 3815.
[2] Present address: Department of Biological Sciences, University of Denver, Denver, CO 80208.
* To whom correspondence should be addressed.

with digitonin, decomposition of the digitonide on the steam bath with pyridine and extraction of the dry residue with benzene to remove the sterols. The sterols were identified by comparing them to authentic standards by gas chromatography (GC) (Varian 3700, 2 m, 2 mm inner dimension column, 5% OV-101 on Anachrom ABS, 275 C; peak areas estimated by triangulation) and by TLC of the steryl acetates on $AgNO_3$ coated sigel plates with CH_2Cl_2.

RESULTS AND DISCUSSION

Selective uptake of sterols was tested under axenic conditions with a medium containing 250 mg/l of a 1:2:4 (w/w/w) mixture of cholesterol, campesterol and sitosterol and *D. mojavensis.* The sterols in the medium and in the flies are shown in Figure 1. The ratio of the 3 sterols in the flies, 1:1.7:0.8, showed that cholesterol and campesterol were adsorbed selectively over sitosterol by the insects.

In order to compare this result with natural conditions, 3 species of *Drosophila* were reared from their natural host cacti in the laboratory as described in the previous section. Saguaro cactus *(Carnegiea gigantea)* was used for rearing *D. nigrospiracula*, organ-pipe cactus *(Stenocereus thurberi)* was used with *D. mojavensis* and agria cactus *(Stenocereus gummosis)* was used with *D. arizonensis* and *D. mojavensis* (21).

The results from the saguaro-*D. nigrospiracula* combination are shown in Figure 2. As fresh saguaro (Fig. 2A) decays, the yeasts living in the tissue contribute ergosterol to the nonsaponifiable fraction of the decayed plant (Fig. 2B), but the *Drosophila* larvae discriminate against these sterols, stigmasterol and sitosterol, in favor of campesterol (Fig. 2C). The small peak in Figure 2C with the relative retention time (RRT) of cholesterol is present as a shoulder on the comparably larger peaks in Figures 2A and 2B. Lipids represent ca. 2-3% of the dry weight of decayed saguaro and the sterols are ca. 5% of the lipid fraction (Kircher, unpublished). The peak area ratios of campesterol to sitosterol were 1:3.2 for fresh saguaro, 1:3 for the decaying tissues and 1:0.7 for *D. nigrospiracula*, which shows a strong discrimination against the uptake of sitosterol by the insects from their natural substrate.

The sterols present in *D. mojavensis* reared from decaying organ pipe cactus are shown in Figure 3B. The insects are able to take up organ pipe phytosterols (Fig. 3A) against a large concentration gradient of other lipids. The nonsaponifiable fraction (Fig. 3C), which represents ca. 2/3 of the 10% lipids present in organ

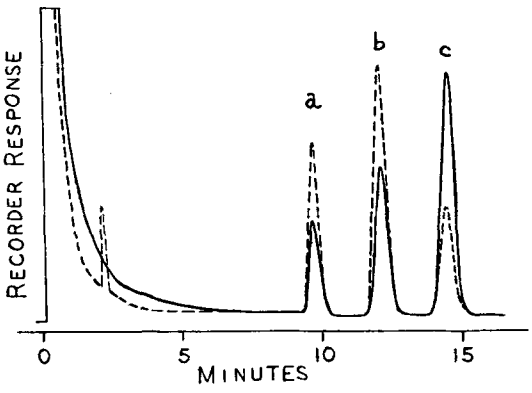

FIG. 1. *D. mojavensis* reared axenically from a medium containing a 1:2:4 mixture of cholesterol (a), campesterol (b), and sitosterol (c). Sterols in medium ———. Sterols in *D. mojavensis* – – – –.

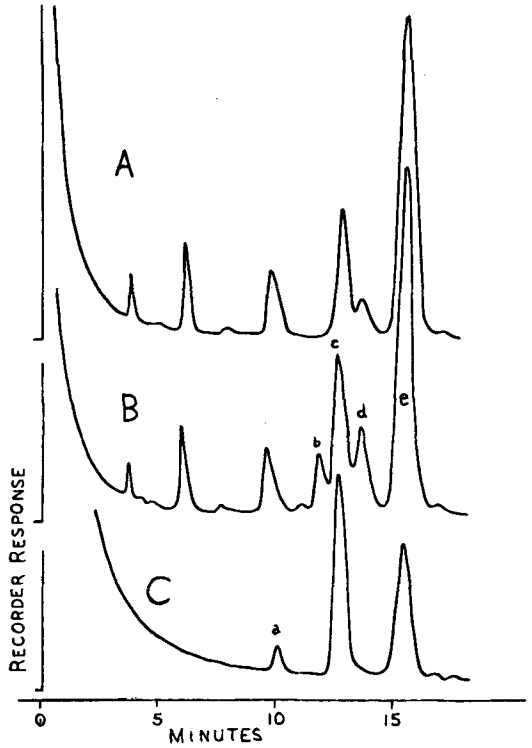

FIG. 2. *D. nigrospiracula* reared from decaying saguaro cactus. A. Nonsaponifiable fraction of fresh saguaro. B. Nonsaponifiable fraction of decayed saguaro. C. Sterols in *D. nigrospiracula.* (a) Cholesterol(?), (b) ergosterol, (c) campesterol, (d) stigmasterol, (e) sitosterol.

pipe cactus (dry wt basis), is composed principally of 3β,6α-sterol diols and 2 pentacyclic triterpene diols. The phytosterols are only 0.9% of the nonsaponifiable fraction (22). Even so, just as in the 2 cases above, *D. mo-*

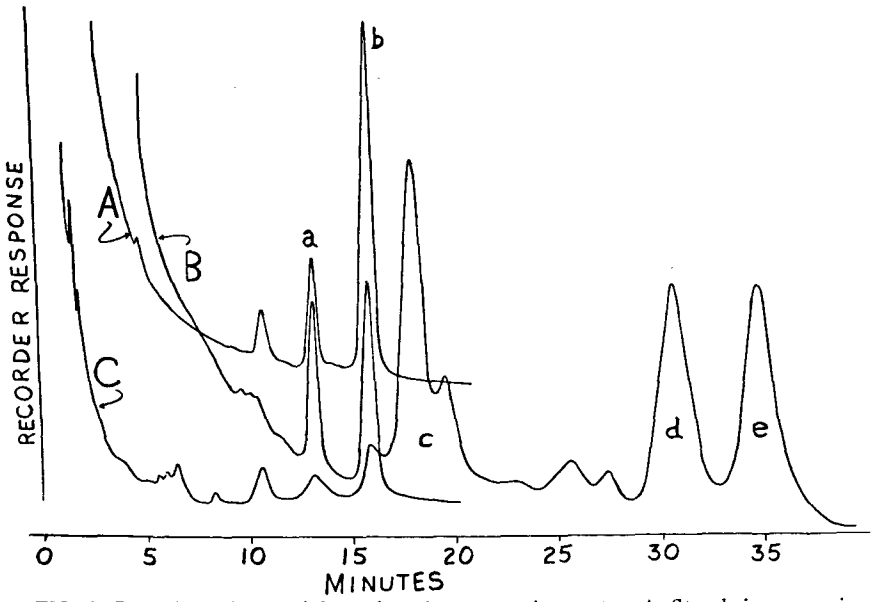

FIG. 3. *D. mojavensis* reared from decaying organ pipe cactus. A. Sterols in organ-pipe cactus phytosterol fraction. B. Sterols in *D. mojavensis*. C. Nonsaponifiable fraction of decaying organ-pipe cactus. (a) Campesterol, (b) sitosterol, (c) sterol diols, (d) calenduladiol, (e) betulin.

javensis discriminates against sitosterol; the campesterol-sitosterol peak area ratio was 1:4 for the cactus and 1:1.6 for the fly sterols.

In the last example of selective uptake of sterols, *D. arizonensis* and *D. mojavensis* were individually reared from decaying agria cactus. The phytosterols have not yet been isolated from this plant. The nonsaponifiable fraction contains sterol diols, 2 triterpene diols and a triterpene triol. Sitosterol is barely visible in a GC separation diagram of this fraction (Fig. 4C). Nevertheless, both species of *Drosophila* selectively absorb the phytosterols from the agria lipids (ca. 8% of the dry wt of the tissue) with a campesterol-sitosterol peak area ratio of 1:2.7 for the *D. arizonensis* (Fig. 4A) and 1:2.4 for the *D. mojavensis* (Fig. 4B) sterols.

Experiments to determine whether or not the dealkylation of phytosterols to cholesterol occurs in *Drosophila* were performed with *D. mojavensis* and *D. mettleri*. The 2 species were reared axenically on a sterol-deficient medium supplemented with 250 mg/l purified sitosterol (Fig. 5A). The minor peak, with the retention time of cholesterol that was present in the GC separation diagrams of the sterols from both species of *Drosophila* (Figs. 5B, 5C), may represent selective uptake of this sterol from trace quantities in the medium. In both cases, campesterol, present as an impurity in the original sitosterol, has a relatively greater proportion of the sterols in the flies than in the medium and, again, demonstrates the selective absorption of this sterol in preference to sitosterol.

The absence of significant quantities of cholesterol in 4 species of *Drosophila* that have either been fed sitosterol under axenic conditions or the mixture of campesterol and sitosterol occurring in their natural host plants, taken together with the inability of these insects to utilize desmosterol, an intermediate in the sitosterol and campesterol dealkylation to cholesterol, is strong evidence that *Drosophila* are unable to remove methyl or ethyl groups from ingested phytosterols. Whether this property is unique to cactophilic *Drosophila* or is extended over the whole genus remains to be seen.

ACKNOWLEDGMENTS

We thank the National Science Foundation for Grant DEB-8207056 to support this work and Jane McCreary for technical assistance.

REFERENCES

1. Thompson, M.J., Louloudes, S.J., Robbins, W.E., Waters, J.A., Steele, J.A., and Mosettig, E. (1963) J. Insect Physiol. 9, 615-622.
2. Clayton, R.B., Hinkle, P.C., Smith, D.A., and Edwards, A.M. (1964) Comp. Biochem. Physiol. 11, 333-350.
3. Svoboda, J.A., Nair, A.M.G., Agarwal, N., and Robbins, W.E. (1979) Experientia 35, 1454-

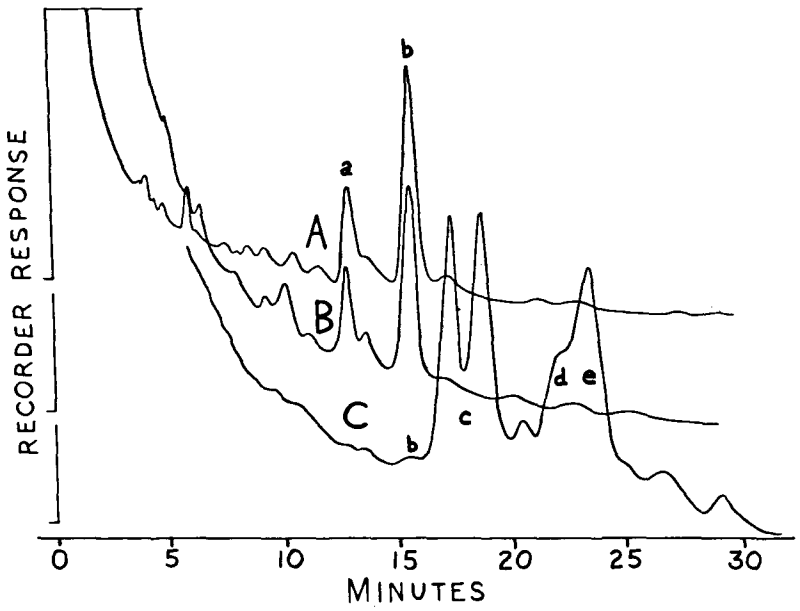

FIG. 4. *D. arizonensis* and *D. mojavensis* reared from decaying agria cactus. A. Sterols in *D. arizonensis*. B. Sterols in *D. mojavensis*. C. Nonsaponifiable fraction of decaying agria cactus. (a) Campesterol, (b) sitosterol, (c) sterol diols, (d) maniladiol, (e) erythrodiol.

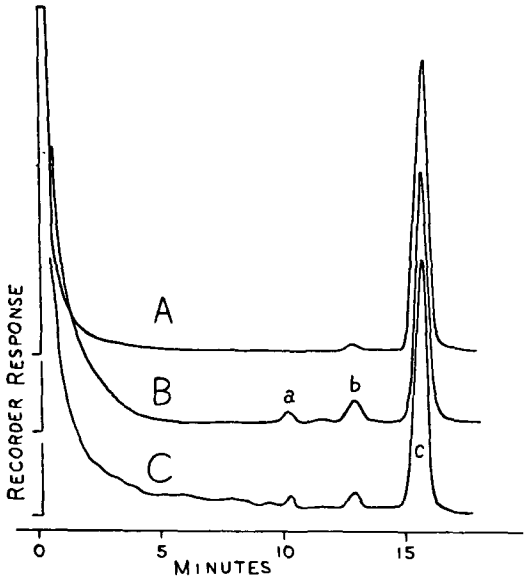

FIG. 5. *D. mojavensis* and *D. mettleri* reared from a sterol-deficient medium supplemented with sitosterol. A. Sitosterol added to the medium. B. Sterols in *D. mojavensis*. C. Sterols in *D. mettleri*. (a) Cholesterol(?), (b) campesterol.

1455.
4. Svoboda, J.A., Dutky, S.R., Robbins, W.E., and Kaplanis, J.N. (1977) Lipids 12, 318-321.
5. Kircher, H.W. (1982) in Cholesterol Systems in Insects and Animals (Dupont, J., ed.) Chap. 1, p. 20, CRC Press, Boca Raton, FL.
6. Nicotra, F., Ronchetti, F., and Russo, G. (1982) Lipids 17, 184-186.
7. Campbell, B.C., and Nes, W.D. (1983) J. Insect Physiol. 29, 149-156.
8. Svoboda, J.A., Thompson, M.J., Herbert, E.W., Jr., Shortino, T.J., and Szczepanik-Vanleeuwen, P.A. (1982) Lipids 17, 220-225.
9. Denneulin, J.Cl., and Lamy, M. (1982) Experientia 38, 800-801.
10. Al-Izzi, M.A.J., and Hopkins, T.L. (1982) Comp. Biochem. Physiol. 71B, 637-641.
11. Ritter, K.S., Weiss, B.A., Norrbom, A.L., and Nes, W.R. (1982) Comp. Biochem. Physiol. 71B, 345-349.
12. Van't Hoog, E.G. (1936) Z. Vitaminforsch. 5, 118-125.
13. Heed, W.B., and Kircher, H.W. (1965) Science 149, 758-761.
14. Goodnight, K.C., and Kircher, H.W. (1971) Lipids 6, 166-169.
15. Kircher, H.W., and Gray, M.A. (1978) J. Insect Physiol. 24, 555-559.
16. Kircher, H.W., Phariss, R.L., Rosenstein, F.U., Baldwin, D., and Fogleman, J.C. (1982) Lipids 17, 209-214.
17. Kircher, H.W., Rosenstein, F.U., and Fogleman, J.C. (1984) Lipids 19, 231-234.
18. Cooke, J., and Sang, J.H. (1970) J. Insect Physiol. 16, 801-812.
19. Bos, M., Burnet, B., Farrow, R., and Woods, R.A. (1976) Genet. Res. 28, 163-176.
20. Thompson, M.J., Svoboda, J.A., Kaplanis, J.N., and Robbins, W.E. (1972) Proc. R. Soc. Lond. B 180, 203-221.
21. Fellows, D.P., and Heed, W.B. (1972) Ecology 53, 850-858.
22. Kircher, H.W. (1980) Phytochemistry 19, 2707-2712.

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Incorporation of Fatty Acids into Phospholipids in L Cells Stimulated by Antibody

WILLIAM T. SHEARER* and **ROBERT G. ULRICH**, *Departments of Pediatrics and of Microbiology and Immunology, Baylor College of Medicine and the Allergy and Immunology Service, Texas Children's Hospital, Houston, TX 77030*

ABSTRACT

Binding antibodies to surface membranes stimulated incorporation of fatty acids (FA) into phospholipids of L cells. Antibodies stimulated at least a 3.4-fold greater incorporation of arachidonic acid into phosphatidylinositol than into any other class of phospholipid when compared on a molar basis ($p < 0.003$). This enhanced incorpoation was selective, depending on the character of the FA, because antibodies stimulated the incorporation of arachidonic acid at least 2.4-fold more than oleic acid, palmitic acid or stearic acid ($p < 0.001$). Surprisingly, an antibody-stimulated incorporation of palmitic acid into sphingomyelin (SM) was at least 2.2-fold greater than that into any other class of phospholipid ($p < 0.001$) and the antibody-stimulated incorporation of palmitic acid into SM was at least 60-fold greater than that of arachidonic acid, stearic or oleic acid ($p < 0.001$). Nontoxic doses of ethylenediamine tetraacetic acid (EDTA), dexamethasone, 4-bromophenacylbromide and indomethacin inhibited the antibody-stimulated incorporation of arachidonic acid into cellular phospholipids, principally phosphatidylinositol (PI), and similarly inhibited the antibody stimulation of DNA synthesis. We conclude that when antibody binds to surface antigens on L cells, a rapid and selective incorporation of fatty acids into certain cellular phospholipids occurs, possibly mediated by calcium-dependent phospholipases. Degradation products of arachidonic acid, i.e., prostaglandins, may be important in these antibody stimulation events, as well. These early changes in phospholipid metabolism may serve as an important signal or mechanism for the subsequent stimulation of DNA synthesis in L cells. *Lipids*, 19:239-249, 1984.

INTRODUCTION

The importance of the turnover of membrane phospholipids in cell activation processes is rapidly becoming apparent in several areas of biology and medicine. In every system examined, there are common elements: binding of ligand to cell surface receptors, associated membrane fluxes of calcium, selective turnover of various chemical moieties of membrane phospholipids principally phosphatidylinositol (PI), and resulting cell secretion or performance of physiological function. Moreover, in many cell systems, the final cell secretion process is preceded by a modulation of arachidonic acid metabolites such as prostaglandins or leukotrienes. Several examples of activated cell systems that display this calcium-phospholipid effect are thrombin-activated platelets (1), zymosan-activated neutrophils (2), anti-IgE challenged mast cells (3), corticotropin-stimulated adrenocortical cells (4), lectin-activated lymphocytes (5), growth factor-treated normal 3T3 cells (6) and bradykinin-stimulated transformed 3T3 cells (7).

Working in a model transformed cell line system and attempting to explain certain aspects of the immunological enhancement of tumor cell growth, we have been able to observe a direct stimulating effect of either specific or heterospecific antibodies on cell growth in vitro (8) or in vivo (9). Complement activated through its third component via the classical complement pathway augmented these processes (10). An early influx of calcium (11) preceded an observable antibody-dependent increased labeling of ^{32}P in the isolated membrane PI fraction (12). More recently, we have been able to document an antibody induced increase in incorporation of arachidonic acid in the PI fraction of cellular phospholipids (13).

The present experiments examined the fatty acid specificity of this antibody-dependent stimulated incorporation and correlated the early perturbations in phospholipid metabolism with subsequent changes in nucleic acid metabolism.

MATERIALS AND METHODS

Cell Culture

A methylcholanthrene transformed mouse fibroblast cell line, L cell (14), was maintained in Falcon 75 cm^2 tissue culture flasks (Becton Dickinson and Co., Oxnard, CA) with minimum essential media (Eagle, GIBCO, Grand Island, NY) containing 10% fetal calf serum and bicarbonate buffer. Cell cultures were incubated at 37 C in a 5% CO_2, humid air incubator.

*To whom correspondence should be addressed.

Materials

The following radiochemicals used were purchased from Amersham (Arlington Heights, IL): [1-^{14}C]arachidonic acid (58.4 mCi/mmol), [1-^{14}C]stearic acid (55.7 mCi/mmol), [9,10(n)-^{3}H]oleic acid (5.7 Ci/mmol), [9,10(n)-^{3}H]-palmitic acid (500 mCi/mmol), and [methyl-^{3}H]thymidine (50 Ci/mmol). Dexamethasone, indomethacin, 4-bromophenacyl bromide and ethylenediamine tetraacetic acid (EDTA) were obtained from Sigma Chemical Co. (St. Louis, MO). Methyl ester standards of free fatty acids (FFA) for gas liquid chromatography (GLC) were purchased from NuChek Prep, Inc. (Elysian, MN). Phospholipid standards for thin layer chromatography (TLC) were purchased from Avanti Biochemicals, Inc. (Birmingham, AL). High purity organic solvents were obtained from Burdick and Jackson Laboratories (Muskegon, MI) and reagent grade glacial acetic acid from Fisher (Fair Lawn, NJ). All TLC performed used Redi Plate silica gel G precoated 250 μM plates from Fisher Scientific Co. (Houston, TX).

Antiserums

Rabbit antimouse L cell sera (AL) and control (adjuvant-injected) rabbit sera (CR) were produced as previously described (12). Two AL preparations were made: AL No. 1 stimulated fatty acid incorporation best at a 5% concentration (by volume); AL No. 2, with a higher antibody titer, stimulated best at a 0.3% concentration (by volume). Both antisera had prominent specificity for a Mr = 42,000 surface antigen that has been identified as actin (15, Shearer et al., unpublished observations).

FFA Analysis by GLC

Total lipid extraction was made of the nutrient medium surrounding the L cells during radioactive fatty acid uptake experiments (see below). That is, the nutrient medium contained Eagle's minimum essential medium, 10% fetal calf serum and 5% rabbit serum (either CR or AL). Lipids were extracted at room temperature for 15 min with 2, 5 ml volumes of chloroform/methanol (2:1) and 1, 5 ml volume of chloroform/methanol (1:2). The addition of 1 ml 0.1 M NaCl during the extractions enhanced the solvent interface formation. The chloroform fractions were pooled and dried under a stream of anhydrous nitrogen gas.

The total lipid extractions of the nutrient medium were taken up in small volumes of chloroform and applied as bands on Silica Gel G TLC plates (Fisher Scientific Co.), dried under nitrogen and chromatographed in 1 dimension using the solvent system: iso-octane/diethyl ether/acetic acid (75:25:2). Areas of the TLC plates identified by comigration with a mixture of standard FFA were visualized with a distilled water mist, scraped from the plates, placed in 15 ml glass tubes with Teflon lined caps, extracted from the silica gel by 3, 3 ml volumes of chloroform/methanol (2:1) and dried under a stream of anhydrous nitrogen gas. This method of Morrisett et al. (16) effectively separates FFA from phospholipids and neutral lipids.

Methyl ester derivatives of the isolated FFA were made by adding 1 ml each of BF_3-methanol and methanolic base (Supelco, Bellefonte, PA) and boiling the sealed tubes in a heated water bath for 2 min. The tubes were cooled, 1 ml of BF_3-methanol was added and the sealed tubes were again boiled for 3 min. Methyl esters were taken up in 3, 3 ml washes of hexane that were pooled and dried under a stream of anhydrous nitrogen gas. GLC analyses were run on a Hewlett Packard 5830A instrument with a model 18850A programmer-integrator using a 6 ft glass column, i.d. = 2 mm, packed with 10% Silar 10C on Supelcoport 100/120 mesh (Supelco). GLC-run parameters were: 20 ml/min nitrogen flow rate, injection port and flame ionization detector (FID) temperatures of 285 C. Sample solvent for injection was hexane or carbon disulfide. Fatty acid methyl esters were quantitated relative to the amount of standards added, in proportion to the corresponding GLC peak areas. Methyl ester standards of the major fatty acids (combined in an equal weight percentage) were used, with 3.8-5.5 average nmol detected.

Radioactive-labeled fatty acids purified by preparative TLC were added to replicate starting samples before extraction with chloroform-methanol and radioactivities of the final methyl esters were measured using a liquid scintillation counter. Extraction and isolation efficiencies were comparable for oleic, palmitic, stearic and arachidonic acids when measured independently, and were ca. 90%. The FFA of intracellular lipids of 4×10^7 L cells (100 times the number used in uptake experiments) were analyzed using identical methods.

Specific Activities of Fatty Acids

The final radioactivities of the fatty acids in the nutrient medium surrounding the cells were: [^{14}C]arachidonic acid 0.50 μCi/ml, [^{14}C]stearic acid 0.65 μCi/ml, [^{3}H]oleic acid 2.5 μCi/ml, and [^{3}H]palmitic acid 2.5 μCi/ml. Using the data obtained by GLC, we calculated the total amounts of FFA in 1 ml of the nutrient medium (containing the isotopes) sur-

rounding the cells during an experiment to be arachidonic acid 11.5 nmol, stearic acid 30 nmol, oleic acid 30 nmol and palmitic acid 55 nmol (no isotope effects were assumed). We also determined that the amounts of these fatty acids in the intracellular pools of the number of L cells used in an experiment, i.e., 4×10^5, was $\leq 1.5\%$ of the amounts present in 1 ml of the nutrient medium. This value agreed well with our calculations for the amount of intracellular FFA in L cells, using the data of Weinstein et al., who analyzed the lipids of L cells grown in the same nutrient medium (17). Therefore, intracellular pools of FFA were not used in calculating the specific activities of the FFA.

Radioactive Fatty Acid Uptake Studies

Two methods of uptake were used. In Method 1, single cell suspensions were prepared by treating L cell monolayers with a 0.05% trypsin solution, washing the centrifuged cells in nutrient medium and resuspending cells in nutrient medium at a cell density of 2 to 3×10^4 cells/ml. The cells were allowed to grow in 10 ml volumes in tissue culture tubes incubated at 37 C for 22 hr, at which time the cell density was 4 to 6×10^4 cells/ml. The cell cultures were centrifuged at $800 \times g$ for 5 min at 4 C, 9 ml of supernatant were removed and the cells were resuspended in the remaining 1 ml of nutrient medium. To start the experiment, AL No. 1 and CR (both 5% by vol) were added to replicate sets of cell cultures, radioactive fatty acids were added as small volumes (10 or 20 μl) of solvent free suspensions (organic solvent dried under N_2) vortexed in Ca^{++}- and Mg^{++}-free Hanks balanced salt solution or as suspensions in 12% bovine serum albumin, phosphate buffered saline (pH 7.4). The cultures were incubated for various time periods at 37 C. To stop the experiment, 4 ml of ice-cold 10% trichloroacetic acid were added and the cell precipitates were extracted for 15 min at room temperature in 2 ml of chloroform/methanol (1:2) as previously described (12). Between 3.7% and 9.4% (average 6.7%) of the added radioactivity was recovered in the chloroform/methanol extract. In Method 2, AL No. 2 and CR (both 0.3% by vol) were incubated in cell cultures for 24 hr at 37 C before the addition of radioactive fatty acids, incubated at 37 C for 1 additional hour and harvest as described above.

Label Release Studies

Cells were grown for 24 hr as described above in the presence of [^{14}C]arachidonic acid (0.5 μCi/ml), centrifuged at 800 g, washed with nutrient medium, resuspended in nutrient medium containing 5% CR or AL No. 1 (both 5% by vol), incubated for various time periods and analyzed for retained radioactivity in isolated phospholipids, as described above.

Phospholipid Analysis by TLC

Chloroform-methanol extracts were vortexed for 10 sec with 2 ml of 0.1 M NaCl at 4 C and the chloroform layer was either used immediately for TLC or stored for up to 48 hr at −80 C with a nitrogen overlayer. Resolution of the major classes of phospholipids, including PI, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), sphingomyelin (SM) and lysophosphatidylethanolamine, lyphosphatidylinositol and lysophosphatidylcholine was achieved by either of 2 solvent systems. The first was composed of chloroform/methanol/distilled water (130:50:8) for the first dimension and chloroform/methanol/glacial acetic acid/0.1 M sodium borate (75:45:12:4.5) for the second dimension (4). The second solvent system was chloroform/methanol/acetic acid/0.9% NaCl (150:75:24:12) for the first dimension and chloroform/methanol/acetic acid/0.9% NaCl (300:45:48:12.5) for the second dimension (18). TLC spots were visualized (plates sprayed with 5% sulfuric acid in ethanol and lightly charred at 110 C for 30 min), scraped, and counted for radioactivity in a Searle Isocap/300 liquid scintillation counter (Searle Analytic Inc., Des Plaines, IL). Phospholipid identities were determined by comigration with high purity reference standards. In 10 experiments, the average percentage recovery of total radioactivity ([^{14}C]-arachidonic acid) was 90% (range 70-107%).

Percentage Labeling of Phospholipids by Fatty Acids

The percentage labeling of phospholipids by fatty acids was calculated by the following formula: pmol fatty acid incorporated/pmol phospholipid isolated $\times$ 100. The pmol of isolated classes of L cell phospholipids were taken from the measurements previously performed by this laboratory (12). In 4×10^5 L cells, 387 pmol of PI, 3,510 pmol of PC, 1,650 pmol of PE, 426 pmol of PS and 465 pmol of SM were found. An average molecular weight of 775 was used for the phospholipids.

Autoradiogram of [^{14}C] Arachidonic Acid and [^{3}H] Palmitic Acid Labeled Phospholipids

After separating the phospholipids, the dried gel plate was sprayed with a radioactivity enhancer (New England Nuclear, Boston, MA) and exposed to Kodak XAR5 film for 2 weeks.

[^{3}H]Thymidine Incorporation into DNA in the Presence of a Calcium Chelator or Enzyme Inhibitors

Cells were harvested from monolayers by a 2 min exposure to a 0.05% trypsin solution and 2×10^5 cells were placed in tissue culture tubes in 1 ml of nutrient medium containing either CR or AL No. 2 at a final volume of 0.3% (stimulation occurred from 0.1% to 5% volumes but was best at 0.3%). The calcium inhibitor was EDTA and the enzyme inhibitors were dexamethasone (19), 4-bromophenylacyl bromide (20) and indomethacin (21). Chemical inhibitors were placed at various concentrations in replicate sets of cell cultures and the cells were incubated at 37 C for 24 hr, the last 6 hr of which [^{3}H]dThd was present in the medium at a final radioactivity concentration of 2.5 μCi/ml. Cell cultures were harvested by washing with phosphate buffered saline over polycarbonate filters (1 μ pore size, Nucleopore Corp., Pleasanton, CA) with the aid of a suction manifold. Retained radioactivity has been previously shown to be contained in nuclear DNA (22).

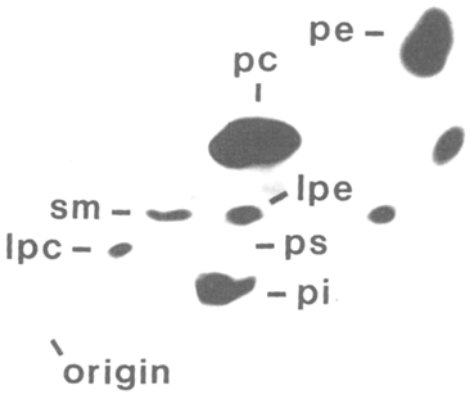

FIG. 1. Two-dimensional TLC autoradiograph of L cell phospholipid extraction. The first solvent system (150:75:24:12 chloroform/methanol/acetic acid/H_2O) ran from left to right and the second (300:45:48:12.5 chloroform/methanol/acetic acid/H_2O) from the bottom to the top of the figure. $CHCl_3$-CH_3OH extraction of 1.4×10^6 cells was incubated 1 hr with 1.4 μCi [^{14}C]arachidonic acid and 13 μCi [^{3}H]-palmitic acid. The abbreviations used are: PI, phosphatidylinositol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SM, sphingomyelin; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine. Lysophosphatidylinositol (present between the origin and PI) did not reproduce well on the photograph and is not labeled with an abbreviation. Unlabeled spots are unidentified lipids.

[^{14}C]Arachidonic Acid Uptake Studies in the Presence of a Calcium Chelator or Enzyme Inhibitors

[^{14}C]Arachidonic acid uptake studies were performed as described above (Method 2) in the presence of 100 μM EDTA and adjusted to pH 7.4. Experiments were also performed in the presence of 2 phospholipase inhibitors: dexamethasone (0.025 μM) (19) and 4-bromophenylacyl bromide (0.40 μM) (20) and a cyclooxygenase inhibitor, indomethacin (10 μM) (21). Immediately before adding to cell cultures, the inhibitors were dissolved in ethanol and the solutions were diluted with Ca^{++}- and Mg^{++}-free Hank's solution. Ethanol blanks were also added to controls for a final concentration in culture of less than 0.2% ethanol. Cells were incubated with either CR or AL No. 2 (both 0.3% by vol) in the presence of inhibitors for 24 hr before the addition of radioactive fatty acid, incubated at 37 C for an additional 1 hr and harvested. At these concentrations of chemical inhibitors, no toxic effects on cell viability or replication in either CR or AL cell cultures were observed as measured by Trypan blue dye exclusion.

Statistical Analysis of Data

The independent Student's *t* test was used to assess the statistical significance of observed differences.

RESULTS

Autoradiogram of Separated L Cell Phospholipids

A visual example of the separation of individual classes of phospholipids of L cells labeled with [^{14}C]arachidonic acid and [^{3}H]palmitic acid is presented in an autoradiogram in Figure 1. All of the major phospholipids are identified as well as the lyso compounds of PC and PE. In quantitative studies presented below, only incorporations into PI, PC, PE, PS and SM are recorded.

Determination of FFA in Nutrient Medium

Table 1 lists the percentage of composition and total amount of FFA in the nonradioactive nutrient medium surrounding the L cells during radioactive fatty acid incorporation. In addition, we analyzed the FFA composition of undiluted fetal calf serum and found it to be very similar to that reported by Spector et al. (23). For example, we found the percentage of fatty acid compositions of fetal calf serum to contain 24.3 ± 0.4% palmitic, 18.5 ± 0.6% stearic, 30.8 ± 3.8 oleic and 10.7 ± 1.2 arachidonic acids. The percentage of fatty acid composition of the nutrient medium containing 10% fetal

TABLE 1

FFA in Nonradioactive Medium Surrounding L Cells During Radioactive Fatty Acid Uptake Experiments

Fatty acid[a]	Percentage of composition[b]		nmol/ml[b]	
	NM[c]	NM + 5% RS[d]	NM[c]	NM + 5% RS[d]
14:0 (myristic acid)	15.0 ± 2.8	15.5 ± 2	29.0 ± 3.8	38 ± 2
14:1 (myristoleic acid)	11.8 ± 0.7	11.3 ± 0.1	23.1 ± 2.7	28 ± 2
16:0 (palmitic acid)	23.4 ± 1.0	22.2 ± 0.9	38.3 ± 3.6	50 ± 2
16:1 (palmitoleic acid)	8.4 ± 0.5	6.7 ± 0.1	11.9 ± 2.2	14 ± 1
18:0 (stearic acid)	9.0 ± 1.0	8.4 ± 0.5	11.8 ± 2.2	18 ± 1
18:1 (oleic acid)	12.2 ± 0.8	13.2 ± 1.5	17.1 ± 0.4	30 ± 6
18:2 (linoleic acid)	4.6 ± 0.6	8.6 ± 0.9	7.0 ± 0.1	17 ± 1
18:3 (linolenic acid)	3.7 ± 0.5[e]	3.1 ± 0.5	6.1 ± 1.1[e]	5.6 ± 0.8
20:4 (arachidonic acid)	1.5 ± 0.1	1.6 ± 0.1	2.6 ± 0.1	3.0 ± 0.1

[a]Minor fatty acids omitted.

[b]Mean ± standard error of 3 determinations.

[c]NM, nutrient medium was Eagle's minimum essential medium + 10% fetal calf serum.

[d]RS, rabbit serum.

[e]Mean ± standard error of 2 determinations.

calf serum yielded substantially different values for stearic, oleic and arachidonic acids, which emphasizes the importance of analyzing the actual medium the cells are tested in. The addition of 5% rabbit serum to the nutrient medium did not change the percentage of distribution of fatty acids but increased the pmol recovered.

Time Dependence of [^{14}C] Arachidonic Acid Incorporation into L Cell Phospholipids

The early time dependence of [^{14}C]arachidonic acid incorporation into L cell phospholipids in cells acutely stimulated by AL was determined (Table 2). Using only the phospholipids with the largest incorporations, we recorded statistically significant AL-enhanced [^{14}C]arachidonic acid incorporation at 30, 60 and 120 min. At 60 min, [^{14}C]arachidonic acid incorporations into PI (53%), PE (28%), and PC (18%) were maximally enhanced. Reflecting the mass amounts of the phospholipids in L cells (see Materials and Methods), the greatest baseline (CR) incorporation and the greatest absolute increase in incorporation (AL-CR) of [^{14}C]arachidonic acid took place in PC. But when the incorporations were computed on a molar basis (i.e., percentage of phospholipid labeled by fatty acid), the greatest baseline incorporation and the greatest increased incorporation of [^{14}C]arachidonic acid occurred in PI, reflecting its rapid metabolism. Subsequent early incorporations of fatty acids were performed at 60 min of incubation with AL.

Release of [^{14}C] Arachidonic Acid from Prelabeled L Cell Phospholipids

The release of [^{14}C]arachidonic acid from phospholipids in prelabeled L cells acutely stimulated by AL was measured (Table 3). We measured the release of [^{14}C]arachidonic acid over the same time intervals, during which significant increases in incorporation because of AL were recorded, i.e., up to 120 min of stimulation by AL (see Table 2). Considering the 15 min time values as a baseline, no significant differences of release caused by AL appeared by 60 min, but rather, only nonspecific release (8 to 14%) of [^{14}C]arachidonic acid occurred during the 45 (60-15) min interval in all phospholipids (only one difference was statistically significant caused by the small differences). As well as nonspecific release of [^{14}C]arachidonic acid from phospholipids, significant differences in release specifically caused by AL appeared by 120 min. For example, 45% of [^{14}C]arachidonic acid was released from PI in AL-stimulated cells compared with a 23% release in control (CR) cells in the 105 (120-15) min interval. That the radioactivity released from cells represented compounds other than arachidonic acid secondary to metabolic interconversion during the 24 hr preincubation is possible. In 2 separate experiments, we determined that CR itself did not cause a release of [^{14}C]arachidonic acids from phospholipids in prelabeled cells (data not shown).

Selectivity of Incorporation of Radioactive Fatty Acids into Phospholipids

When L cells were treated with AL, a rapid (1 hr) increased incorporation of fatty acids into certain cellular phospholipids (Figs. 2 and 3) occurred. Each fatty acid was incorporated in a distinctive pattern. The greatest absolute

TABLE 2

Time Dependence of [^{14}C]Arachidonic Acid Incorporation into Phospholipids in L Cells Stimulated by AL

	Phosphatidylinositol (PI)		Phosphatidylcholine (PC)		Phosphatidylethanolamine (PE)	
	pmol	% PI labeled	pmol	% PC labeled	pmol	% PE labeled
15 min						
CR	14.3 ± 0.6	3.7	58.7 ± 5.5	1.7	12.8 ± 1.4	0.8
AL	13.2 ± 1.4	3.4	63.5 ± 3.0	1.8	14.6 ± 1.4	0.9
30 min						
CR	15.1 ± 1.4	3.9	109 ± 5.7	3.1	19.3 ± 1.5	1.2
AL	23.6 ± 1.8[a]	6.1	120 ± 6.4	3.4	23.8 ± 1.0[a]	1.4
60 min						
CR	34.0 ± 2.6	8.8	193 ± 8.2	5.5	41.4 ± 3.0	2.5
AL	52.3 ± 5.4[a]	13.5	228 ± 3.9[a]	6.5	52.4 ± 3.1[a]	3.2
120 min						
CR	54.1 ± 2.6	14.0	277 ± 7.1	7.9	70.7 ± 2.7	4.3
AL	70.2 ± 6.0[a]	18.1	333 ±19.8[a]	9.5	90.8 ± 3.6[a]	5.5

As described in Materials and Methods, cells were preincubated at 37 C for 24 hr before the addition of 5% CR or AL No. 1 and [^{14}C]arachidonic acid (0.5 μCi/ml) and additional incubation at 37 C for various periods of time. Results are expressed as the means ± standard errors of the pmol of arachidonic acid incorporated into isolated phospholipids and as the percentage of phospholipid labeled by arachidonic acid (i.e., pmol arachidonic acid incorporated/pmol phospholipid X 100). Two experiments were performed in duplicate and 1 experiment was performed in triplicate (total data points = 7).

[a]$P < 0.04$ for differences between AL and corresponding CR.

TABLE 3

Release of [^{14}C] Arachidonic Acid from Phospholipids in Prelabeled L Cells Stimulated by AL

	Phosphatidylinositol (PI)		Phosphatidylcholine (PC)		Phosphatidylethanolamine (PE)	
	pmol	% PI labeled	pmol	% PC labeled	pmol	% PE labeled
15 min						
CR	145 ± 14	35.4	1,102 ± 46	31.4	607 ± 29	36.8
AL	146 ± 17	37.7	1,118 ± 50	31.8	623 ± 24	37.7
60 min						
CR	127 ± 7	32.8	963 ± 81	27.4	567 ± 51	34.3
AL	135 ± 8	34.9	965 ± 18[a]	27.5	566 ± 23	34.3
120 min						
CR	112 ± 7[a]	29.0	815 ± 14[a]	23.2	499 ± 12[a]	30.2
AL	81 ± 2[a,b]	20.8	679 ± 30[a,b]	19.3	389 ± 27[a,b]	23.6

As described in Materials and Methods, cells were incubated at 37 C in the presence of [^{14}C] arachidonic acid (0.5 μCi/ml) for 24 hr, washed, resuspended in nonradioactive nutrient medium containing 5% CR or AL No. 1 and incubated at 37 C for 15, 60 or 120 min. Results are expressed as the means ± standard errors of the pmol of arachidonic acid contained in isolated phospholipids and as the percentage of phospholipid labeled (see legend to Table 2 for explanation). Three experiments were performed in triplicate and 2 experiments were performed in duplicate (total data points = 13).

[a] $P < 0.04$ for differences between corresponding values at 15 min.

[b] $P < 0.02$ for differences between AL and CR at 120 min.

increase in the percentage of phospholipid labeled with fatty acid in the presence of AL took place with SM labeled with palmitic acid (7.2%). The baseline (CR) labeling of SM with palmitic acid was also unique because no other fatty acid was appreciably incorporated into SM. The next greatest absolute increase in labeling phospholipid in the presence of AL occurred with arachidonic acid in PI (6.1%), which was at least 2.4-fold greater than the increase in labeling of PI with oleic acid or palmitic acid in the presence of AL. The incorporation of stearic acid, which is known to be predominantly bound in O-acyl linkage to the C1 of the glycerol backbone of PI in mammalian tissues (24), was not stimulated by AL, despite a substantial baseline (CR) incorporation. This contrasts with the AL-dependent incorporation of arachidonic acid, which is known to be the predominant fatty acid attached to C2 of the glycerol moiety of PI (24). Considering the total amounts of the phospholipids, substantial AL dependent increases in labeling of PC and PE with arachidonic, oleic and palmitic acids were found as well.

DNA Synthesis in AL Stimulated Cells: Effects of Calcium Chelator and Enzyme Inhibitors

As noted previously (22), AL significantly stimulated the incorporation of radioactive thymidine into DNA measured at 24 hr (Fig. 4). The calcium chelator, EDTA, and enzyme inhibitors were tested over wide concentration ranges for effects on AL-stimulated DNA synthesis. Each reagent produced inhibition of DNA synthesis in L cells, particularly those stimulated by AL. At lower concentrations of the inhibitors, a selective effect on the DNA synthesis in AL-stimulated cells was apparent, e.g., 0.025 μM dexamethasone, 0.4 μM 4-bromophenacylbromide, 10 μM indomethacin and 100 μM EDTA. Assessing all concentrations of the inhibitors, including those where baseline (CR) values were inhibited, the absolute decreases in DNA synthesis were much more pronounced in AL-stimulated cell cultures.

[^{14}C] Arachidonic Acid Incorporation into Phospholipids of AL Stimulated Cells: Effects of a Calcium Chelator and Enzyme Inhibitors

Because suspecting that enzymes might be operating in the AL-stimulated incorporation of fatty acids into phospholipids is reasonable, as suggested in several other stimulus-cell activation systems (1-7), experiments were performed in the presence of EDTA, dexamethasone, 4-bromophenacylbromide and indomethacin (Fig. 5). Phospholipase A_2 (25) and phospholipase C (26) are both activated by calcium and chelation of calcium by EDTA prevents their activation. In every instance, the AL-stimulated increase in the incorporation of arachidonic acid into PI, PC and PE was significantly inhibited ($p < 0.001$). EDTA (100 μM), dexamethasone (0.025 μM), bromophenacylbromide (0.40 μM) and indomethacin (10 μM) specifically decreased the AL-responsive arachidonic acid incorporation into phospholipids without

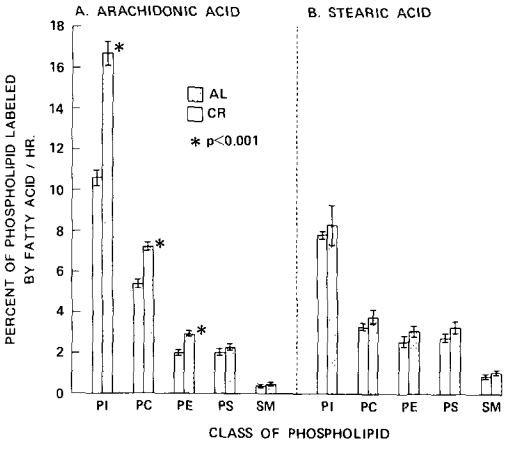

FIG. 2. Incorporation of radioactive fatty acids into phospholipids of L cells treated with 5% CR or AL No. 1 for 1 hr; A, arachidonic acid; B, stearic acid. The results are expressed as the mean percentages ± standard errors of isolated phospholipids labeled with radioactive fatty acids as described in the Materials and Methods section calculated for 4×10^5 cells. Results for arachidonic acid ([^{14}C]arachidonic acid, 0.5 μCi/ml) represent 9 experiments performed in triplicate (total 27 data points): 3 experiments were performed simultaneously with 3 separate experiments with stearic acid ([^{14}C]stearic acid, 0.65 μCi/ml), 3 experiments were performed in the same sets of cell cultures with oleic acid ([^{3}H]oleic acid, 2.5 μCi/ml) seen in Figure 3A, 3 experiments were performed in the same set of cell cultures with palmitic acid ([^{3}H]-palmitic acid, 2.5 μCi/ml) seen in Figure 3B. The AL stimulated incorporation of arachidonic acid was statistically significant for PI, PC and PE as indicated on the figure. The absolute increased percentage labeling for PI with arachidonic acid in the presence of AL was statistically significant compared with that for PC or PE with a p value of <0.001. Also, the absolute increased percentage labeling of PI with arachidonic acid in the presence of AL was statistically significant compared with labeling with stearic acid (Fig. 2B), oleic acid (Fig. 3A) or palmitic acid (Fig. 3B) with a p value of <0.001.

significantly affecting the baseline (CR) incorporations.

These concentrations of the inhibitors were well below those reported to cause nonspecific effects in other biological systems. For example, Hofmann et al. (27) have shown that higher concentrations of 4-bromophenylacyl bromide (30-100 μM) not only inhibit phospholipase C activity in platelets but affect other biological activities as well. Jesse and Franson (28) found that the cyclooxygenase inhibitor indomethacin at 100 μM was capable of significantly inhibiting platelet phospholipase A_2 activity. Rittenhouse-Simmons (29) reported that 70 μM indomethacin-inhibited platelet diglyceride lipase

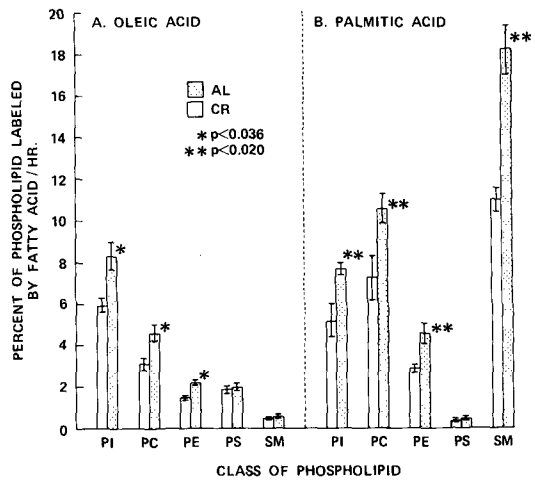

FIG. 3. Incorporation of radioactive fatty acids into phospholipids of L cells treated with 5% CR or AL No. 1 for 1 hr; A, oleic acid; B, palmitic acid. See legend to Figure 2 for description of experiments. The AL-stimulated incorporation of oleic acid and palmitic acid into isolated phospholipids was statistically significant (see figure for p values). The absolute increased labeling for SM with palmitic acid in the presence of AL was statistically significant compared with that for PI, PC or PE with a p value of <0.003. Also, the absolute increased percentage labeling of SM with palmitic acid was statistically significant compared with labeling with any other fatty acid tested ($p < 0.001$).

activity. Hial et al. found that 100 μM indomethacin inhibited cell replication of rat hepatoma and human fibroblast cell lines (30).

DISCUSSION

These investigations have broadened the spectrum of cell membrane ligands capable of stimulating incorporation of arachidonic acid in membrane phospholipids to include IgG antibodies with specificity for surface antigens on transformed tumor cell lines. Antibodies stimulated a selective incorporation of arachidonic acid in PI, which was at least 2.4-fold greater than the incorporation of palmitic, oleic or stearic acids in PI. Specificity of the class of phospholipid also occurred because, when compared on a molar basis, incorporation of arachidonic acid in PI was at least 3.4-fold greater than that in any other phospholipid. Although the precise enzymatic steps involved in the antibody enhanced incorporation of arachidonic acid into cellular phospholipids are not clear, rapid enzymatic deacylation with phospholipases followed by rapid reacylation of fatty acids with acyltransferases into phospholipids seem to be involved, as they are with

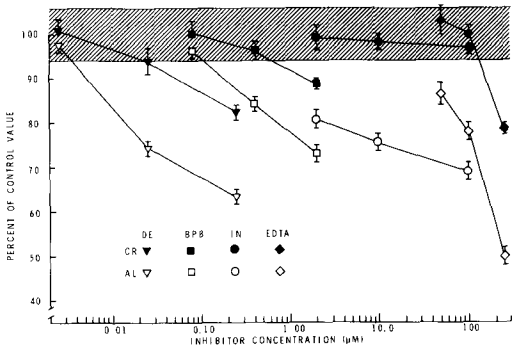

FIG. 4. Incorporation of radioactive thymidine into DNA of L cells treated with 0.3% CR, or AL No. 2 for 24 hr: effects of a calcium chelator and enzyme inhibitors. The results are expressed as the percentage of control values of 12 experiments performed in sextuplicate (n = 72). The mean control values ± standard error of the means (cpm of retained radioactivity) for CR and AL cell cultures were 24,784 ± 1,329 and 96,936 ± 6,235. The standard error of the means for the control values are indicated by the area within the shaded area. Three experiments (performed in sextuplicate, 18 total data points per inhibitor condition) were performed and compared with control CR and AL cell cultures performed simultaneously. Open points represent AL cell cultures and solid points represent CR cell cultures in the presence of the inhibitors indicated on the figure. Whenever the standard-error spread of inhibitor values (brackets) fall outside the standard-error spread of the corresponding control values, a p value of at least <0.05 exists for the difference. The abbreviations used are: DE, dexamethasone; BPB, 4-bromophenacyl-bromide; IN, indomethacin.

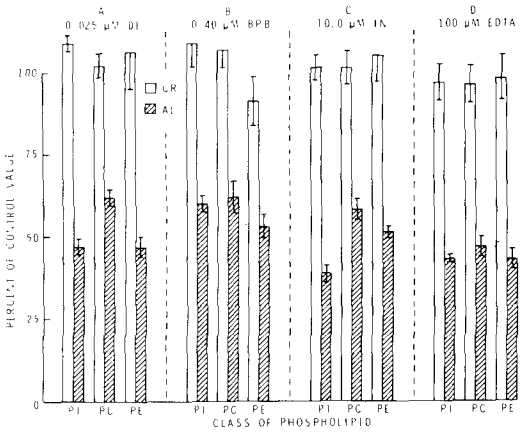

FIG. 5. Incorporation of [^{14}C]arachidonic acid into selected phospholipids of L cells treated with 0.3% CR or AL No. 2 for 24 hr: inhibition with a calcium chelator and enzyme inhibitors. The results are expressed as the percentage of control values of 4 experiments performed in triplicate (n = 12). The control mean values ± the standard error of the means of CR and AL for PI were 120 ± 5.5 and 239 ± 8.0 pmol/ hr; these values correspond to 31.0 ± 1.4 and 61.8 ± 2.1% labeling of PI. The control mean values of CR and AL for PC were 279 ± 8.8 and 539 ± 10.8 pmol/hr; these values correspond to 8.0 ± 0.3 and 15.4 ± 0.3% labeling of PC. The control mean values of CR and AL for PE were 174 ± 13.5 and 323 ± 18 pmol/hr; these values correspond to 10.5 ± 0.8 and 19.6 ± 1.2% labeling of PE. For all differences between appropriate CR and AL values, $p < 0.001$. EDTA (100 μM), dexamethasone (DE) (0.025 μM), 4-bromophenacylbromide (BPB) (0.40 μM) or indomethacin (IN) (10 μM) were included in replicate sets of experiments. At these concentrations of reagents, the values for CR cell cultures were unchanged, whereas the values for the AL cell cultures were significantly reduced ($p < 0.001$).

other stimulus-cell activation systems (1-7,31). Release studies with [^{14}C]arachidonic acid in the presence of antibodies support a deacylation-reacylation mechanism. Also, de novo synthesis of phospholipids cannot account for the incorporation as we have previously shown that no change occurs in the glycerol labeling of phospholipids in antibody stimulated cells (13). In addition, the total phospholipid content of antibody-stimulated cells was shown by us not to increase over a 2 hr time interval (12).

A phospholipase seems to be involved in the antibody-enhanced incorporation of arachidonic acid in membrane phospholipids because deprivation of calcium by a chelator inhibited the effect and nontoxic concentrations of putative phospholipase inhibitors also prevented an augmented incorporation of arachidonic acid. In other cell systems, dexamethasone inhibits phospholipase indirectly through the induction of protein inhibitors (32). Because early increased uptake of calcium in these antibody-stimulated cells precedes or occurs simultaneously (11), believing that the sudden influx of calcium produced by the binding of antibody to the cell membrane activates phospholipase A_2, which deacylates arachidonic acid from the number 2 carbon of the glycerol moiety of PI is reasonable. That calcium activates phospholipase C, which enzymatically forms 1,2-diacylglycerol, followed by release of arachidonic acid by the enzymatic action of diglyceride lipase (33) is also possible. Turnover of arachidonic acid in membrane phospholipids, especially PI, suggests an important role for phospholipases, especially phospholipase A_2, in many types of cell activation (34).

Possibly the liberation of arachidonic acid by phospholipase A_2 provides a substrate for enzymatic productions of prostaglandins, which themselves exert control over cellular functions. The fact that a nontoxic concentration of indomethacin inhibited the antibody-induced augmentation of [^{14}C]arachidonic acid turnover in PI suggests that cyclooxygenase conversion of released arachidonic acid to prostaglandins is an important subsequent metabolic step in antibody stimulation of transformed cell lines. Experiments with other transformed mouse cell lines have documented that production of PGE_2 is derived via cyclooxygenase conversion of arachidonic acid released predominantly from PI in response to membrane perturbation by ligands (7,35). In the mouse transformed cell line described in this report, similar enzymatic production of prostaglandins is probably being modulated by the binding of antibody molecules to surface membrane structures. The patterns of inhibition of DNA synthesis in L cells stimulated by antibody in the presence of a calcium chelator and enzyme inhibitors at 24 hr mimic, for the most part, the inhibition seen in the antibody stimulation of fatty acids into cellular phospholipids at 24 hr. This implies that changes in phospholipid metabolism are an important part of the cell stimulation process, possibly mediated by the conversion of arachidonic acid into prostaglandins.

The unexpected, enhanced incorporation of palmitic acid into SM in response to stimulation of cells by antibody suggests that enzymatic deacylation-reacylation reactions may be a more generalized consequence of the binding of immunoglobulin molecules to cell surface antigens. Palmitic acid has been found to be the most prevalent fatty acid in SM in the Ehrlich tumor cell line (36). Although our findings imply that SM is rapidly deacylated and reacylated with palmitic acid when IgG ligands bind to cell membrane antigens, that de novo synthesis of SM occurs is possible. However, previously we were unable to detect an increase in total SM under similar conditions (12). Under certain conditions, Rode et al. (37) observed a stimulated incorporation of palmitic acid into SM in concanavalin A stimulated lymphocytes. Together, these observations suggest an active role for palmitic acid turnover in SM in cells activated by protein ligands.

In summary, these investigations suggest that binding high affinity antibodies to cell surface antigens activates enzymatic processes, resulting in the deacylation and reacylation of arachidonic acid in PI and palmitic acid in SM. The enzymes responsible for the effects remain to be conclusively elucidated. The rapid and specific incorporation of arachidonic acid into certain phospholipids, principally PI, seems to be an important biochemical event in the antibody stimulation of these cells, which precedes DNA synthesis and cell growth.

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REFERENCES

1. Lapetina, E.G., Billah, M.M., and Cuatrecasas, P. (1981) Nature 292, 367-369.
2. Walsh, C.E., Waite, B.M., Thomas, M.J., and DeChatelet, L.R. (1981) J. Biol. Chem. 256, 7228-7234.
3. Kennerly, D.A., Sullivan, T.J., and Parker, C.W. (1979) J. Immunol. 122, 152-159.
4. Schrey, M.P., and Rubin, R.P. (1979) J. Biol. Chem. 254, 11234-11241.
5. Parker, C.W., Kelly, J.P., Falkenhein, S.F., and Huber, M.G. (1979) J. Exp. Med. 149, 1487-1503.
6. Habenicht, A.J.R., Glomset, J.A., King, W.C., Nist, C., Mitchell, C.D., and Ross, R. (1981) J. Biol. Chem. 256, 12329-12335.
7. Hong, S.L., and Deykin, D. (1981) J. Biol. Chem. 256, 5215-5219.
8. Shearer, W.T., Philpott, G.W., and Parker, C.W. (1973) Science 182, 1357-1359.
9. Fink, M.P., Parker, C.W., and Shearer, W.T. (1975) Nature 255, 404-405.
10. Shearer, W.T., Atkinson, J.P., Frank, M.M., and Parker, C.W. (1975) J. Exp. Med. 141, 736-752.
11. Shearer, W.T., Atkinson, J.P., and Parker, C.W. (1976) J. Immunol. 177, 973-980.
12. Shearer, W.T., and Crouch, J.A. (1977) J. Immunol. 119, 911-917.
13. Shearer, W.T., and Richards, J.E. (1981) Biochem. Biophys. Res. Commun. 101, 800-806.
14. Nettleship, A., Earle, W.R. and Shelton, E. (1943) J. Natl. Cancer Inst. 4, 229-234.
15. McClure, J.A., Bryan, J., Brinkley, B.R., and Shearer, W.T. (1983) Fed. Proc. 42, 917.
16. Morrisett, J.D., Pownall, H.J., Jackson, R.L., Segura, R., Gotto, Jr., A.M., and Taunton, O.D. (1977) in Polyunsaturated Fatty Acids (Kunau, W.H. and Holman, R.T., eds.) pp. 139-161, American Oil Chemists' Society, Champaign, IL.
17. Weinstein, D.B., Marsh, J.B., Glick, M.C., and Warren, L.W. (1968) J. Biol. Chem. 244, 4103-4111.
18. Anderson, R.E., Maude, M.B., and Feldman, G.L. (1969) Biochim. Biophys. Acta 187, 345-353.
19. Roberts, M.F., Deems, R.A., Mincey, T.C., and Dennis, E.A. (1977) J. Biol. Chem. 252, 2405-2411.
20. Blackwell, G.J., Flower, R.J., Nijkamp, F.P., and Vane, J.R. (1978) Br. J. Pharmac. 62, 79-89.
21. Hamberg, M., and Samuelsson, B. (1974) Proc. Natl. Acad. Sci. USA 71, 3400-3404.
22. Shearer, W.T., Philpott, G.W., and Parker, C.W. (1975) Cell. Immunol. 17, 447-462.
23. Spector, A.A., Mathur, S.N., Kaduce, T.L., and Hyman, B.T. (1981) Prog. Lipid Res. 19, 155-186.
24. Holub, B.J., Kuksis, A., and Thompson, W.

(1970) J. Lipid Res. 11, 558.
25. Brockerhoff, H., and Jensen, R.G. (1974) Lipolytic Enzymes, pp. 194-265, Academic Press, New York.
26. Hofmann, S.L., and Majerus, P.W. (1982) J. Biol. Chem. 257, 6461-6469.
27. Hofmann, S.L., Prescott, S.M., and Majerus, P.W. (1982) Arch. Biochem. Biophys. 215, 237-244.
28. Jesse, R.L., and Franson, R.C. (1979) Biochim. Biophys. Acta 575, 467-470.
29. Rittenhouse-Simmons, S. (1980) J. Biol. Chem. 255, 2259-2262.
30. Hial, V., DeMello, M.C.F., Horakova, Z., and Beaven, M.A. (1977) J. Pharm. Exp. Ther. 202, 446-454.
31. Irvine, R.F. (1982) Biochem. J. 204, 3-16.
32. Hirata, F., Schiffman, E., Venkatasubramanian, K., Solomon, D., and Axelrod, J. (1980) Proc. Natl. Acad. Sci. USA 77, 2533-2536.
33. Bell, R.L., Kennerly, D.A., Stanford, N., and Majerus, P.W. (1979) Proc. Natl. Acad. Sci. USA 76, 3238-3241.
34. Flower, R.J. (1981) in The Prostaglandin System (Berti, F. and Velo, G.P., eds.), pp. 27-37, Plenum Press, New York.
35. Schremmer, J.M., Blank, M.L., and Wykle, R.L. (1979) Prostaglandins 18, 491-504.
36. Kuksis, A. (1978) Handbook of Lipid Research (Kuksis, A., ed.), Vol. 1, pp. 381-422, Plenum Press, New York.
37. Rode, H.N., Szamel, M., Schneider, S., and Resch, K. (1982) Biochim. Biophys. Acta 688, 66-74.

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Influence of Culture Filtrate of *Trichoderma viride* and Barley on Lipid Metabolism of Laying Hens [1,2]

ASAF A. QURESHI,[a*] NEVILLE PRENTICE,[a] ZAFEER Z. DIN,[a] WARREN C. BURGER,[a] CHARLES E. ELSON[b] and MILTON L. SUNDE,[c] [a]*USDA-ARS, Cereal Crops Research Unit, 501 N. Walnut Street, and* [a]*Department of Agronomy,* [b]*Department of Nutrition and* [c]*Department of Poultry Science, University of Wisconsin, Madison, WI 53706*

ABSTRACT

The suppression of hepatic 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase and cholesterol 7 α-hydroxylase, previously noted in studies of the influences of barley and the filtrate of *Trichoderma viride* culture (CF) on cholesterol metabolism in sexually immature birds, is shown in sexually mature birds. Barley, CF or both were fed in one study from the day of hatching, in another during the period of sexual maturation and, in a third study, CF was fed to mature layers. CF suppressed HMG CoA reductase by 30-50% and cholesterol 7 α-hydroxylase by 32-45% when added to the control diet. In birds fed barley rather than corn, the respective activities were 25-36% and 24-31% lower. These effects were expressed in the lowering of plasma cholesterol by 11-36%. Lipogenic activity based on the assays of 4 enzymes was increased 2-3 times by the treatments and plasma triglyceride elevated by 12-86%. The start of egg production by birds fed CF preceded the controls by 17 days. Birds fed barley trailed controls by 11-14 days. CF countered the barley-conditioned delay. Egg yolk cholesterol concentrations were lowered by both treatments. Eggs produced by hens fed barley were lower in weight; CF increased egg and yolk weights. Tissues from birds fed CF or barley for up to 30 weeks appeared to be normal.

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INTRODUCTION

In short-term studies of the effects of corn- and barley-based diets on avian lipid metabolism, the latter grain was shown to suppress cholesterolopoieses and enhance lipogenesis when fed to 1-day-old chicks for 21 days. Weight gain by birds fed the barley-based diet was also suppressed. The addition of a crude enzyme preparation, cellulase Onazuka, which is rich in β-glucanase activity, to the barley-based diet improved weight performance but not to the level achieved with corn. Cellulase Onazuka, the lyophilized filtrate of *Trichoderma viride* cultures (CF), suppressed cholesterolopoiesis and stimulated lipogenesis when added to the corn-based diet (1). These effects of barley and of CF on enzyme activities were reproduced in a swine study, also 21 days long. Barley was the equal of corn in supporting weight gain by 5-month-old swine (2). In the chicken study, only acetyl CoA carboxylase and fatty acid synthetase activities were measured to provide a basis for estimates of lipogenic activity. In the swine study, activities of glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, malic enzyme and citrate cleavage enzyme were also examined. The latter 2 enzymes responded in parallel with changes in acetyl CoA carboxylase and fatty acid synthetase activities (2). These studies raise 2 issues, which are addressed in this report: are the treatment effects influenced by the age of the bird, and are the treatment effects maintained over the long term? Because cholesterol levels in circulation and in avian liver and porcine muscle were reduced by these treatments (1,2), the suppression of avian cholesterolopoiesis might also result in the production of "low" cholesterol eggs.

MATERIALS AND METHODS

Sources of chemicals, substrates, labeled substrates, enzymes, diagnostic kits and cellulase Onazuka were identified in preceding publications (1,2). White Leghorn females were used in these studies. For the first study, 1-day-old chicks were purchased from a local hatchery. The 12-week-old chicks used in the second study were provided by the University of Wisconsin Poultry Research Laboratory. Laying hens 56 weeks old, used for the third experiment, were taken from the laying flock maintained by the Poultry Research Laboratory.

[1] Cooperative investigation between the Science and Education Administration, USDA, and the College of Agricultural and Life Sciences, University of Wisconsin-Madison. A preliminary report of this work was presented at the 67th Annual Meeting of the Federation of American Societies for Experimental Biology, Chicago, IL, April 10-15, 1983.

[2] Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the USDA and does not imply its approval to the exclusion of other products that may also be suitable.

* To whom correspondence should be addressed.

TABLE 1

Experimental Design

Diet	Age(wks)	Grain									
		Study 1[a]				Study 2[a]				Study 3[b]	
		Corn		Barley		Corn		Barley		Corn	
		CF[c]		CF[c]		CF[c]		CF[c]		CF[c]	
Grower	0 – 12	0	50	0	50	———		———	[e]	———	[e]
Grower	13 – 18	0	50	0	50	0	50	0	50	———	[e]
Layer	19 – 24	0	100	0	100	0	100[f]	0	100[f]	———	[e]
Layer	25 – 30	0	100	0	100					———	[e]
Layer	31 – 56									———	[e]
Layer	57 – 60									0	100[g]

[a]n = 12/group.
[b]n = 10/group.
[c]mg lyophilyzed CF of *T. viride*.
[d]All birds were fed the corn-based grower ration (Table 2) for 12 weeks and then the grower and layer diets formulated as shown on Table 2.
[e]All birds were fed for commercial egg production using rations formulated at the University of Wisconsin Poultry Research Laboratory. The layer diet given in Table 6 was fed for 4 weeks.
[f]Eggs collected from 21-week-old hens.
[g]Eggs collected from 59-week-old hens.

The design of the experiment is given in Table 1. For the investigation of the long-term treatment responses (study 1), the birds were fed for 18 weeks a grower diet based either on corn or barley with or without 50 mg CF/kg diet and then for 12 weeks a layer diet based on corn or barley with or without 100 mg CF/kg diet. Compositions of these diets are given on Table 2. Determinations of nitrogen, diethyl ether extractables, ash and starch of corn, barley and of the grower diets were carried out as described in "Cereal Laboratory Methods" (3). The estimations of acid detergent fibers (4) and of β-glucan, xylan and araban were carried out as described previously (5,6).

The second study was an examination of the effects of the application of the treatments during the period of sexual maturation. The chicks were fed the grower diets for 6 weeks and then for 6 weeks the layer diets described in Table 2. The third study addresses only the effect of the CF on the lipid metabolism of mature hens. The composition of the layer diet used in that study is presented in the summary table.

In studies 1 and 2, the chicks were reared with continuous illumination for the duration of the grower dietary period. The layer diets were fed with 14-hr artificial light. Diets and water were provided ad libitum. Feed disappearance and weight gain were recorded at weekly intervals. Age at the onset of laying (studies 1 and 2) and egg production were recorded. Eggs were collected in studies 1 and 2 for the 4 consecutive days when the birds reached 23 weeks of age. Eggs were collected during the final week of the third study at which time the hens were 60 weeks of age.

The eggs were weighed and their yolks separated and cleaned over cheesecloth. Each yolk was weighed and homogenized in saline solution (1 g/5 ml) with a Polytron for 5 sec. The yolk homogenates were stored in air-tight bottles at −20 C.

At the end of the studies, the birds were weighed and blood samples (3 ml) were collected in tubes containing heparin (500 units/0.3 ml saline solution) for harvesting the plasma, which was stored at −20 C. The birds were then killed and their livers removed, washed and placed on ice.

Liver homogenates were prepared in 0.1 M potassium phosphate buffer, pH 7.4, containing 4 mM magnesium chloride, 1 mM (EDTA) and 2 mM dithiothreitol as described (1,2). The 100,000 × *g* supernatant fractions (cytosol) and precipitates (microsomes) were stored at −20 C until assayed for enzymatic activities.

Cholesterol Studies

Plasma and yolk cholesterol concentrations were estimated using Worthington Cholesterol Reagent kits (1,2). The yolk homogenate (10 μl) was diluted with 1 ml saline for this analysis. The accuracy of the enzymic method was confirmed using the 0-phthalaldehyde procedure described by Rudel and Morris (7). Assays for 3-hydroxy-3-methylglutaryl CoA (HMG CoA) reductase and cholesterol 7 α-hydroxylase, the enzymes generally considered

TABLE 2

Percent Composition of the Chicken Diets

Ingredients	Diets[a]			
	Grower		Layer	
	Corn	Barley	Corn	Barley
Corn[b]	66.5	–	68.5	–
Barley[b]	–	79.0	–	79.5
Soybean meal (44%)	14.9	11.5	20.5	9.5
Meat scrap	5.0	5.0	–	–
Alfalfa meal (17%)	2.0	2.0	2.0	2.0
Dicalcium phosphate	1.0	1.0	1.0	1.0
Calcium carbonate	0.5	0.5	7.0	7.0
Mineral mixture[c]	0.5	0.5	0.5	0.5
Vitamin mixture[d]	0.5	0.5	0.5	0.5

[a]Five percent granite grit was incorporated at the expense of each diet.

[b]Analyses of corn and barley were 10.6 and 12.5% of protein on 'as is' basis.

[c]Contains (per kg): sodium chloride (NaCl), 4.8 mg; zinc sulphate ($ZnSO_4$), 90 mg; manganese dioxide (MnO_2), 110 mg.

[d]Contains (per kg): vitamin A, 2,000 IU; vitamin D_3, 200 ICU; vitamin E, 10 IU; vitamin K_1, 5 mg; choline, 1.3 g; thiamin, 1.8 mg; niacin, 27 mg; riboflavin, 3.6 mg; pyridoxine, 3 mg; calcium-pantothenate, 10.0 mg; vitamin B_{12}, 10 μg, lysine-HCl, 1 g; methionine, 0.72 g.

to be rate-controlling for hepatic cholesterol (8) and bile acid (8,9) syntheses, were carried out as described previously (10,11) with some modification. HMG CoA reductase activity was determined in 150 μl with 300 μg microsomal protein and cholesterol 7 α-hydroxylase activity in 1 ml with 50 μg protein. The assays were incubated for 30 min (HMG CoA reductase) or 15 min (cholesterol 7 α-hydroxylase) at 42 C. Enzyme activity is expressed as nmol mevalonic acid or pmol 7 α-hydroxycholesterol synthesized per mg microsomal protein or per g liver per min.

Lipogenesis

Plasma triglyceride concentrations were estimated with Worthington Triglyceride Reagent kits (1,2). The assay for acetyl CoA carboxylase was that of Craig et al. (12) where the activity reflects nmol malonyl-CoA formed per mg cytosolic protein or per g liver per min. The activities for citrate cleavage enzyme (13), malic enzyme (14) and fatty acid synthetase (15) were assayed spectrophotometrically at 25 C. These assays were monitored at 340 nm with the recorder set for 0.1 A full scale. The reactions in 0.5 ml were initiated by the addition of 25-250 μg cytosolic protein. The absorbance of 1 mM NADPH or NADH is 6.22. Enzyme activities reflect nmol NADPH or NADH ($NADP^+$) oxidized (or reduced) per mg cytosolic protein or μmol per g liver per min.

Cytosolic and microsomal protein concentrations were estimated by a modification of the Biuret method using bovine serum albumin as the standard (16).

Statistical comparisons of results were performed by 1- or 2-way analysis of variance. When a significant treatment effect was indicated by the F test, the differences between the means were analyzed by the LSD test (17).

RESULTS

The proximate analyses of the cereals and of the cereal-based grower diets are given in Table 3. The corn diet provides 24.5% protein and 3 Kcal/g, 33% by protein, 19% by fat and 48% by carbohydrate. The barley diet provides 19.4% protein and 2.4 Kcal/g, 32% by protein, 7% by fat and 61% by carbohydrate. Both diets provide 8 g protein per 100 Kcal. The 10-fold difference in β-glucans is the distinguishing feature, apart from the distribution of the energy components and the caloric density of the 2 diets.

The long-term feeding of CF in combination with corn or barley has no adverse effect as would be detected by a depression of feed intake or of mature weight or by an enlargement of the liver. Barley supported a mature weight (1628 ± 107 g) equal to that supported by corn (1598 ± 117 g). The stimulation of growth noted in young chicks fed CF (1) had no significant effect on mature weight (Table 4). The impact of barley and of CF on cholesterol metabolism and lipogenesis previously noted in 14- and 21-day studies of young chicks (1) are maintained throughout this 210-day feeding

TABLE 3

Proximate Analysis of Corn, Barley and Cereal-based Diets ('As is' Percentage Basis)

Analysis[a]	Corn	Corn diet	Barley	Barley diet
Nitrogen	1.7	3.92	2.0	3.1
Ether extractables	4.0	3.1	2.3	1.9
Ash	1.5	6.3	2.4	6.2
Starch	66.8	36.7	53.6	37.1
Acid detergent fiber	3.3	4.7	6.2	6.2
Araban	2.8	3.2	3.1	3.9
Xylan	7.6	8.9	6.5	10.4
β-Glucan	0.5	0.6	6.0	6.0
Dietary fiber[b]	14.2	17.4	21.8	26.5
Moisture	9.8	9.8	10.5	9.5

[a]Data presented on 'as is' basis after multiple determination.

[b]Dietary fiber = acid detergent fiber + araban + xylan + β-glucan.

TABLE 4

Effect of Cereals and CF of *T. viride* on Weights, Hepatic Enzyme Activities and Plasma and Egg Lipids of 30-Week-Old Hens*

Parameter	Diet							
	Corn (control)		Corn + culture filtrate		Barley		Barley + culture filtrate	
Initial body weight (g)	38.2 ± 3.3[a]		37.5 ± 3.0[a]		38.4 ± 3.7[a]		38.6 ± 3.5[a]	
Final body weight (g)	1598 ± 117[a]	(100)‡‡	1656 ± 94[a]	(104)‡‡	1628 ± 107[a]	(102)‡‡	1753 ± 122[a]	(110)‡‡
Feed consumption (kg)	104.0		106.0		105.2		108.1	
Liver weight (g)	35.8 ± 3.3[a]		38.5 ± 3.5[a]		38.1 ± 3.6[a]		39.9 ± 4.0[a]	
Liver weight (g/kg body wt)	22.4		23.2		22.8		22.8	
3-Hydroxy-3-methylglutaryl-CoA reductase†	1.1 ± 0.2[a]	(100)‡‡	0.6 ± 0.1[b,c]	(55)	0.7 ± 0.1[b]	(64)	0.4 ± 0.1[c]	(36)
Cholesterol 7α-hydroxylase‡	8.7 ± 1.6[a]	(100)‡‡	5.9 ± 1.2[b]	(68)	6.0 ± 0.4[b]	(69)	3.7 ± 0.8[c]	(43)
Plasma cholesterol§	96 ± 7.0[a]	(100)‡‡	70 ± 9.0[b]	(83)	75 ± 11.0[b]	(89)	64 ± 10.0[b]	(77)
Citrate-cleavage enzyme¶	2.4 ± 0.2[a]	(100)‡‡	4.2 ± 0.2[b]	(175)	2.9 ± 0.1[a]	(121)	4.6 ± 0.4[b]	(192)
Acetyl-CoA carboxylase#	28.2 ± 3.0[a]	(100)‡‡	58.3 ± 4.0[b]	(248)	56.2 ± 2.0[b]	(201)	77.4 ± 3.0[c]	(276)
Malic enzyme**	12.2 ± 0.5[a]	(100)‡‡	21.9 ± 1.1[b]	(180)	14.8 ± 1.3[c]	(121)	28.3 ± 1.2[d]	(232)
Fatty acid synthetase††	3.3 ± 1.5[a]	(100)‡‡	17·0 ± 2.1[b]	(515)	12.0 ± 1.7[c]	(364)	18.6 ± 2.0[b]	(564)
Plasma triglycerides§	1172 ± 219[a]	(100)‡‡	1412 ± 332[a]	(120)	1311 ± 285[a]	(112)	1621 ± 345[a]	(138)
First egg (d)	140		123		151		126	
Egg weight (g)	51.5 ± 5.7[a]	(100)‡‡	56.5 ± 3.2[b]	(110)	48.5 ± 2.8[c]	(94)	55.8 ± 3.8[a]	(108)
Yolk weight (g)	15.5 ± 1.6[a]	(100)‡‡	16.7 ± 2.7[a]	(108)	13.8 ± 3.6[a]	(89)	16.5 ± 2.9[a]	(106)
Yolk cholesterol (mg/g)								
Enzymic process	17.6 ± 1.8[a]	(100)‡‡	14.9 ± 1.6[b]	(85)	15.7 ± 1.2[b]	(89)	13.7 ± 1.4[b]	(78)
Chemical Process	18.3 ± 2.1[a]	(100)‡‡	15.4 ± 2.6[b]	(84)	16.5 ± 1.4[b]	(90)	14.6 ± 1.6[b]	(80)

*Feeding period was 30 week; time of killing was 0800; data expressed as mean ± SD; N = 12 chickens per group. †ηmol of mevalonic acid synthesized per minute per gram of liver. ‡ρmol of [^{14}C]cholesterol into [^{14}C]7α-hydroxycholesterol per min per g liver. §mg/100 ml of plasma. ¶μmol of product formed per min per g liver. #ηmol of product formed per min per g liver. **μmol of $NADP^+$ reduced per min per g liver. ††μmol of NADPH oxidized per min per g liver. ‡‡Percentage of respective control activity data are in parentheses. [a-d]Means within a line and without a common supercript letter are significantly different, $P<0.01$.

period. As was shown in short-term studies with swine (2), lipogenic activities in the liver were elevated in birds fed the barley-based diet. Citrate cleavage and malic enzyme activities were 121% of the control activities; acetyl CoA carboxylase (201%) and fatty acid synthetase (364%) activities were increased to greater degree. The addition of CF to the diet caused a similar pattern of elevation in these enzyme activities (Table 4). The suppression of HMG-CoA reductase (64% of control) and cholesterol 7 α-hydroxylase (69% of control) in livers of birds fed barley is also maintained over this 210-day period. Adding CF to the corn-based diet lowered these activities to a similar degree; the effect of adding CF to the barley-based diet appears to be additive to that of barley. HMG CoA reductase activity was 36% of control and cholesterol 7 α-hydroxylase, 43% of control. The additive effect is also apparent in the lowering of the plasma cholesterol by the combined treatments (77% of control).

First eggs appeared 17 and 25 days earlier when CF was fed from the day of hatching with corn- and barley-based diets. Birds fed the barley-based diet produced smaller eggs (94% of control weight). Addition of CF to the diet resulted in the production of heavier eggs. Yolk cholesterol concentrations measured by the enzymatic procedure agreed with those determined chemically. In terms of activity and concentration, the HMG-CoA reductase and plasma cholesterol of the experimental groups were in the order, control > barley > corn + CF > barley + CF. This pattern also was expressed in terms of yolk cholesterol concentration (Table 4).

The responses of the layers to barley and to CF fed during the period of sexual maturation (Table 5) are generally consistent with those observed in birds fed the dietary treatments throughout the 30 weeks of the first study (Table 4). The treatment order follows the aforementioned pattern, control/barley/corn + CF/barley + CF in either < or > the level of the parameter under consideration. At 24 weeks, birds fed the barley diet were somewhat lighter in weight in both the second study (Table 5) and in the first study (data not shown). During the final 6 weeks of the first study, the birds fed the barley diet caught up with the controls (Table 4). When fed the barley from the day of hatching, the onset of egg production was delayed by 11 days (Table 4). A 14-day delay was noted in the second study (Table 5). The addition of CF to the corn-based diet caused the onset of laying to begin 17 days earlier. A similar effect was obtained by adding CF to the barley-based diet (Tables 4,5). Changes in egg weights, yolk weights and yolk cholesterol concentrations (Table 5) were consistent with those previously noted (Table 4).

In the third study, 0.01% CF was added to the diet of 56-week-old layers. After 4 weeks exposure to CF, hepatic HMG CoA reductase and cholesterol 7 α-hydroxylase activities were ca. 70% of control values and plasma cholesterol, 77% of control. Plasma triglycerides were elevated by 36%. Lipogenic enzyme activities were only modestly increased, with the exception of citrate cleavage enzyme, which exhibited 3-fold control activity. Eggs collected from CF-fed layers during the final week of this study were significantly heavier (69.7 ± 2.1 and 62.4 ± 2.9 g). The cholesterol concentration was significantly lower (17.4 ± 0.2 and 18.7 ± 0.2 mg/g yolk) but, because of the increase in yolk weight (20.3 ± 1.1 and 17.2 ± 1.0 g), the CF treatment produced eggs containing more cholesterol (353 and 322 mg) (Table 6).

DISCUSSION

The impact and the duration of the CF treatment on avian cholesterol metabolism are independent of age-related variables. Hepatic HMG CoA reductase and cholesterol 7 α-hydroxylase activities in birds fed CF were 30-50% lower than control values and their plasma cholesterol levels were 20% lower. The CF treatment caused a 15% decrease in the concentration of yolk cholesterol at 24 weeks and a 7% decrease at 60 weeks. Birds given the CF had higher plasma concentrations of triglycerides but with age, the difference in triglyceride concentration decreases. Consistent with the changes in triglyceride levels are the specific activities of the lipogenic enzymes. CF was more effective in stimulating lipogenic activity in the younger birds.

The birds fed barley also had lower plasma cholesterol concentrations. This decrease might be attributed to the lower fat content or to the higher β-glucan content of barley. The undigestible components of human and animal diets have been associated in recent years with reductions in plasma and body tissue cholesterol concentrations by increasing its excretion (18-21). Cholestyramine also increases cholesterol excretion. In animals treated with this binder, hepatic HMG-CoA reductase activity is induced (22). On the basis of these observations, rationalizing an effect of fiber that could be mediated through the suppression of cholesterol biosynthesis is difficult. On the other hand, polar and apolar components of barley, added to control diets at concentrations not in excess of 1%, suppress hepatic HMG-CoA

TABLE 5

Effect of CF of *T. viride* on Weights, Hepatic Enzyme Activities and Plasma and Egg Lipids in 14-Week-Old Hens*

Parameter	Diet							
	Corn (control)		Corn + culture filtrate		Barley		Barley + culture filtrate	
Initial body weight (g)	892 ± 61[a]		883 ± 64[a]		878 ± 60[a]		887 ± 58[a]	
Final body weight (g)	1541 ± 56[a]		1649 ± 60[a]		1390 ± 67[a]		1585 ± 53[a]	
Feed consumption (kg)	52.4		53.1		53.7		55.2	
Liver weight (g)	34.5 ± 3.0[a,b]		36.8 ± 2.0[a]		30.7 ± 2.8[b]		36.0 ± 2.0[a]	
Liver weight (g/kg body wt)	22.4		22.3		22.1		22.7	
3-Hydroxy-3-methylglutaryl-CoA reductase†	1.2 ± 0.1[a]	(100)‡‡	0.6 ± 0.1[b]	(50)‡‡	0.9 ± 0.1[c]	(75)‡‡	0.6 ± 0.1[b]	(50)‡‡
Cholesterol 7α-hydroxylase‡	9.1 ± 1.5[a]	(100)‡‡	5.0 ± 0.8[b]	(55)	6.9 ± 1.2[a]	(76)	4.9 ± 0.4[b]	(54)
Plasma cholesterol §	110 ± 9.0[a]	(100)‡‡	81.7 ± 7.0[b]	(74)	85 ± 8[b]	(77)	70 ± 7.0[b]	(64)
Citrate-cleavage enzyme¶	3.4 ± 0.3[a]	(100)‡‡	3.8 ± 0.3[a]	(115)	3.8 ± 0.3[a]	(115)	4.0 ± 0.3[a]	(121)
Acetyl-CoA carboxylase#	31.0 ± 3.0[a]	(100)‡‡	74.0 ± 4.0[b]	(239)	69.0 ± 3.0[b]	(223)	88.8 ± 5.0[c]	(286)
Malic enzyme**	13.5 ± 1.3[a]	(100)‡‡	16.0 ± 0.5[b]	(119)	14.8 ± 1.0[b]	(110)	17.9 ± 1.6[b]	(123)
Fatty acid synthetase††	3.7 ± 2.0[a]	(100)‡‡	12.8 ± 2.0[b]	(346)	10.3 ± 3.0[b]	(278)	18.2 ± 2.5[c]	(492)
Plasma triglycerides §	1046 ± 185[a]	(100)‡‡	1686 ± 312[b]	(161)	1417 ± 294[b]	(135)	1917 ± 403[b]	(183)
First egg (d)	147		130		161		147	
Egg weight (g)	50.9 ± 4.0[a,b]	(100)‡‡	55.7 ± 3.0[a]	(109)	48.4 ± 3.0[a,b]	(95)	56.2 ± 4.0[a]	(110)
Yolk weight (g)	15.3 ± 2.0[a]	(100)‡‡	16.9 ± 3.0[a]	(116)	13.5 ± 4.0[a]	(88)	16.6 ± 3.0[a]	(108)
Yolk cholesterol (mg/g)	17.8 ± 2.0[a]	(100)‡‡	14.1 ± 2.0[a,b]	(79)	14.9 ± 1.0[a,b]	(83)	12.9 ± 2.0[b]	(72)

*Feeding period was 12 week; time of killing was 0800; data expressed as mean ± SD; N = 12 chickens per group. †ηmol of mevalonic acid synthesized per minute per g liver. ‡ρmol of [^{14}C]cholesterol into [^{14}C]7α-hydroxycholesterol per min per g liver. §mg/100 ml of plasma. ¶μmol of product formed per min per g liver. #ηmol of product formed per min per g liver. **μmol of $NADP^+$ reduced per min per g liver. ††μmol of NADPH oxidized per min per g liver. ‡‡Percentage of respective control activity data are in parentheses. [a-c]Means within a line and without a common supercript letter are significantly different, $P<0.01$.

TABLE 6

Effect of CF of *T. viride* on Liver Weights, Hepatic Enzyme Activities and Plasma and Egg Lipids of 60-Week-Old Laying Hens*

Parameter†	Diet	
	Corn (control)	Corn + culture filtrate
Initial body weight (g)	1736 ± 197	1744 ± 155
Final body weight (g)	1836 ± 213[a]	1883 ± 190[a]
Feed consumption (kg)	26.75	27.30
Liver weight (g)	39.2 ± 2.7[a]	46.3 ± 4.1[a]
Liver weight (g/kg body wt)	21.35	24.58
β-Hydroxy-β-methylglutaryl-CoA reductase‡	0.789 ± 71[a] (100)‡‡	0.552 ± 52[b] (70)‡‡
Cholesterol 7α-hydroxylase §	1.05 ± 0.17[a] (100)‡‡	0.69 ± 0.06[b] (66)
Plasma cholesterol¶	134 ± 13[a] (100)‡‡	103 ± 18[b] (77)
Citrate-cleavage enzyme#	8.5 ± 1.6[a] (100)‡‡	27.4 ± 4.1[b] (322)
Acetyl-CoA carboxylase#	0.62 ± 0.12[a] (100)‡‡	0.91 ± 0.11[b] (147)
Malic enzyme**	185 ± 28[a] (100)‡‡	236 ± 11[b] (128)
Fatty acid synthetase††	89.5 ± 7[a] (100)‡‡	117.8 ± 8[b] (132)
Plasma triglycerides¶	78 ±3.6[a] (100)‡‡	106 ± 3.0[b] (136)
Egg weight (g)	62.4 ± 2.9[a] (100)‡‡	69.7 ± 2.1[b] (112)
Yolk weight (g)	17.2 ± 1.0[a] (100)‡‡	20.3 ± 1.1[b] (118)
Yolk cholesterol (mg/g)	18.7 ± 0.2[a] (100)‡‡	17.4 ± 0.2[b] (93)

*Each diet contains corn–9.5% (70.2%); soybean meal–44% (14.8%); alfalfa meal–17% (1.7%); meat and bone meal (5%); dicalcium phosphate (0.5%); calcium carbonate (6.8%); NaCl (0.5%); vitamin and mineral mixture (0.5%); grit (5%), was incorporated at the expense of feed. Vitamin and mineral mixture contain (per kg feed): vitamin A, 5,000 IU; vitamin D_3, 900 ICU; vitamin B_{12} 0.01 mg; riboflavin, 3 mg; MnO_2, 50 mg; $ZnSO_4$, 110 mg. †Feeding period was 1 month, data expressed as mean + SD; N = 10 chickens per group; ‡ηmol of mevalonic acid synthesized per min per mg microsomal protein. §ρmol of [C^{14}]-cholesterol into [C^{14}]7α-hydroxycholesterol per min per mg microsomal protein. ¶mg/100 ml of plasma. #ηmol of product formed per min per mg cytosolic protein. **ηmol of $NADP^+$ reduced per min per mg cytosolic protein. ††ηmol of NADPH oxidized per min per mg of cytosolic protein. ‡‡Percentage of respective corn activity data. [a,b]Means within a line and without a common superscript are significantly different ($P<0.01$).

reductase by up to 50% and, concomitantly, lower plasma cholesterol during the course of 21-day studies (23).

The overall effects of barley on hepatic cholesterol metabolism and lipogenesis and on plasma lipid concentrations were similar to those of CF. The major departure in the actions of the 2 materials is that effect on egg production. CF added to the control diet advanced the date of first egg and increased the average weight of eggs produced by layers 24 and 60 weeks of age. Replacement of corn with barley caused a delay in the onset of egg production and decreased the average weights of eggs produced by layers 24 weeks of age. CF added to the barley diet reversed these effects of barley on egg production. All treatments lowered the concentration of cholesterol in the yolk. Other treatments, including drugs (24), dietary protein (25), energy level and source (26), vitamin A (27), ascorbic acid (28), vanadium (29) and fiber (30) have been tested. The most effective treatment involves the use of crude but not purified forms of fiber (24,30).

The influence of CF on egg production might be mediated through an estrogenic effect on the hepatic synthesis of vitellin (31,32). Part of the decrease in yolk cholesterol concentration can be traced to the increase of the yolk triglycerides, an increase mediated by vitellin.

One observation requiring further study is that chicks fed the barley diet over the course of 30 weeks consumed 20% less energy and yet matched the 30-week weight of the controls. At 24 weeks, the 20% deficit in energy intake underlies a 10% lower body weight.

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REFERENCES

1. Qureshi, A.A., Burger, W.C., Prentice, N., and Elson, C.E. (1980) J. Nutr. 110, 1473-1478.
2. Qureshi, A.A., Burger, W.C., Elson, C.E., and Benevenga, N.J. (1982) Lipids 17, 924-934.
3. MacMasters, M.M. (1962) Cereal Laboratory Methods (M.M. MacMasters, 7th ed.) published by American Association of Cereal Chemists, Inc., St. Paul, MN.
4. Goering, H.K., and Van Soest, P.J. (1970) Forage Fiber Analyses. Agricultural Handbook, No. 379, ARS, USDA, Washington, DC.
5. Prentice, N., and Refsguard, J.F. (1978) J. Am. Soc. Brew. Chem. 36, 196-200.
6. Prentice, N., Babler, S., and Faber, S. (1980) Cereal Chem. 57, 198-202.
7. Rudel, L.L., and Morris, M.D. (1973) J. Lipid Res. 14, 364-366.
8. Bucher, N.L.R., Overath, P., and Lynen, F. (1960) Biochim. Biophys. Acta 40, 491-501.
9. Danielsson, H., Einarsson, K., and Johansson, G. (1967) Eur. J. Biochem. 2, 44-49.
10. Shapiro, D.J., Norstrom, J.L., Mitchelen, J.J., Rodwell, V.W., and Schimke, R.T. (1974) Biochim. Biophys. Acta 370, 369-377.
11. Carlson, S.E., Mitchell, A.D., and Goldfarb, S. (1978) Biochim. Biophys. Acta 531, 115-124.
12. Craig, M.C., Nepokroeff, C.M., Lakshmanan, M.R., and Porter, J.W. (1972) Arch. Biochem. Biophys. 152, 619-630.
13. Inoue, H., Suzuke, F., Fukunishi, K., Adachi, K., and Takeda, Y. (1966) J. Biochem. 60, 543-553.
14. Hsu, R.H., and Lardy, H.A. (1967) J. Biol. Chem. 245, 520-526.
15. Nepokroeff, C.M., Lakshmanan, M.R., and Porter, J.W. (1975) Methods Enzymol. 35, 45-49.
16. Gornall, A.G., Bardawill, C.J., and David, M.M. (1949) J. Biol. Chem. 177, 751-766.
17. Snedecor, G.W., and Cochran, W.G. (1971) in Statistical Method, 6th ed., pp. 258-298, The Iowa State University Press, Ames, IA.
18. Trowell, H. (1972) Am. J. Clin. Nutr. 25, 926-932.
19. Anderson, J.W., and Chen, W.J.L. (1979) Am. J. Clin. Nutr. 32, 346-363.
20. Jenkins, D.J.A., Reynolds, D., Slavin, B., Leeds, A.R., Jenkins, A.L., and Jepson, E.M. (1980) Am. J. Clin. Nutr. 33, 575-581.
21. Balmer, J., and Zilversmit, B.D. (1974) J. Nutr. 104, 1319-1328.
22. Higgin, M.J.P., Brady, D., and Rudney, H. (1974) Arch. Biochem. Biophys. 163, 271-282.
23. Qureshi, A.A., Burger, W.C., Din, Z.Z., and Elson, C.E. (1982) Am. J. Clin. Nutr. 35, 855 (Abstract).
24. Keshavarz, K. (1976) Poultry Sci. 55, 1077-1083.
25. Edwards, H.M., Jr., Marion, J.E., and Driggers, J.C. (1962) Poultry Sci. 41, 713-717.
26. Palafox, A.L. (1968) Poultry Sci. 47, 1705 (Abstract).
27. Dua, P.N., Dilwaorth, B.C., Day, E.J., and Hill, J.E. (1967) Poultry Sci. 46, 530-531.
28. Nockels, C.F. (1973) Poultry Sci. 52, 373-378.
29. Hafez, Y.S.M., and Kratzer, F.H. (1976) Poultry Sci. 55, 923-926.
30. Turk, D.E., and Barnett, B.D. (1972) Poultry Sci. 51, 1881 (Abstract).
31. Roepke, R.R., and Bushnell, L.D. (1936) J. Immun. 30, 109-113.
32. Vanstone, W.E., Dale, D.G., Oliver, W.F., and Common, R.H. (1957) Can. J. Biochem. 35, 659-665.

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Temperature-Dependent Deacylation of Molecular Species of Phosphatidylcholine by Microsomal Phospholipase A_2 of Thermally Acclimated Rainbow Trout, *Salmo gairdneri* [1]

N.P. NEAS*, and **J.R. HAZEL**, *Department of Zoology, Arizona State University, Tempe, AZ 85287*

ABSTRACT

Using the ratios of kinetic parameters, V/Km, the deacylation of different molecular species of 1-palmitoyl,2-acyl phosphatidylcholine via microsomal phospholipase A_2 (PLA_2) was studied in liver tissue of thermally acclimated rainbow trout (*Salmo gairdneri*). In general, PLA_2 from fish acclimated to cold temperatures showed an order of preference for the acyl moieties of 18:1 > 18:2 > 18:0. Trout acclimated to warm temperatures generally preferred 18:0 PC, but the actual order of preference depended on the temperature of the assays and the presence of endogenous lipids in the enzyme preparation. At 5 C, the particulate (microsomal) enzyme preferred 18:0 > 18:2 > 18:1, but a lipid-free preparation of the enzyme preferred 18:2 > 18:0 > 18:1. At 20 C, particulate enzyme preferred 18:1 > 18:0 > 18:2 but purified enzyme preferred 18:0 > 18:2 > 18:1. Thus, assay temperature and the presence of microsomal lipids had a greater effect on PLA_2 from fish acclimated to warm temperatures than fish acclimated to cold temperatures. The substrate preference of PLA_2 is discussed with reference to the previously observed changes in membrane fatty acid composition that occur with thermal acclimation in rainbow trout.

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INTRODUCTION

In most poikilotherms, exposure to low ambient temperatures results in the incorporation of highly unsaturated fatty acids into membrane phospholipids, an adaptation that presumably conserves approporiate fluidity for optimal membrane function (1). Recent studies of thermally induced phosphatide restructuring have demonstrated the significance of a deacylation-reacylation cycle in the synthesis of appropriate molecular species (2,3). Because enzymes of lipid metabolism typically have broad substrate specificities, thermally modulated shifts in the substrate preferences of these deacylating and reacylating enzymes could potentially represent one point at which membrane lipid composition is regulated.

The reacylating enzyme, acyl CoA:1-acyl-*sn*-glycero-3-phosphorylcholine acyltransferase, has been demonstrated in microsomes of rainbow trout liver (4), and was shown to prefer oleoyl-CoA over palmitoyl-CoA substrates regardless of the temperature of the assay or the acclimation history of the fish.

Deacylating enzymes, the phospholipases, have also been identified in trout tissues (5,6). Hazel (7) reported that most of the fatty acid substitutions occurring as a result of thermal acclimation were at the *sn*-2 carbon of the major membrane phosphatides, thereby implicating phospholipase A_2 in remodeling of trout cell membranes. Phospholipase A_2 was identified in trout liver microsomes (5) and, following acclimation, exhibited perfect compensation for temperature in both particulate (microsomal) and lipid-free preparations when assayed with 1-acyl,2-oleoyl phosphatidylcholine as substrate (8). The enzyme, however, has not been previously studied for thermally modulated substrate preference.

To further explain the enzymatic basis for the synthesis of appropriate molecular species of phosphatidylcholines at different temperatures in rainbow trout, we have studied the kinetic characteristics of the deacylation of exogenous 1-palmitoyl phosphatidylcholines containing different 18-carbon acyl chains at the *sn*-2 position. Both particulate microsomes and lipid-free preparations from liver tissue of trout acclimated to warm and cold temperatures were assayed at different temperatures to determine the influence of membrane lipids on the substrate preference of the enzyme.

MATERIALS AND METHODS

Materials

1-Palmitoyl lysophosphatidylcholine and dipalmitoyl phosphatidylcholine were obtained from Sigma Chemical Co., St. Louis, MO. 1-[^{14}C]stearic (56.5 mCi/mmol), 1-[^{3}H]oleic (53 mCi/mmol) and 1-[^{14}C]linoleic (52 mCi/mmol) acids were purchased from Amersham

*To whom correspondence should be addressed. Present Address: Department of Biology, Colgate University, Hamilton, NY 13346.

Corp., Arlington Heights, IL. 1-Palmitoyl,2-linoleoyl phosphatidylcholine was obtained from PL Biochemicals, Inc., Milwaukee, WI. Enzymatic grade ammonium sulfate was from Nutritional Biochemicals Corp., Cleveland, OH. Concentrated protein dye reagent was purchased from BioRad Laboratories, Richmond, CA.

Animals

Rainbow trout (*Salmo gairdneri*) were acquired from the Alchesay National Hatchery, Whiteriver, AZ. Fish were maintained in running water at either 5 C or 20 C and a 12L:12D photoregime for at least 1 month before the experiments. They were fed once a day with Glencoe Mills commercial trout food and maintained a healthy appearance and normal activity for the duration of the study.

Substrate Synthesis

Synthesis of 1-palmitoyl,2-[^{14}C]stearoyl phosphatidylcholine (PC), 1-palmitoyl,2-[^{3}H]-oleoyl phosphatidylcholine and 1-palmitoyl,2-[^{14}C]linoleoyl phosphatidylcholine was accomplished using rat liver microsomal acyltransferease to esterify the radiolabeled fatty acids to 1-palmitoyl lysophosphatidylcholine (LPC), as described by Conner et al. (9). Radiolabeled PC was twice purified by thin layer chromatography (TLC) (5). Positional distribution of the radiolabel on PC was determined by snake venom hydrolysis (*Crotalus adamanteus*) according to Dickens and Thompson (10). Over 99% of the radiolabel in the 1-saturated, 2-unsaturated species of PC was esterified at the *sn*-2 position. In the disaturated PC, 90% of the label was esterified to carbon number 2. Substrates were stored in $CHCl_3$/MeOH (2:1, v/v) at -20 C under N_2 and were periodically checked for specific radioactivity by counting aliquots and dividing by molar concentration as determined by phosphorus analysis (11).

Enzyme Preparation

For each experiment, 7 to 9 trout (100-250 g) were decapitated and the livers (0.6-2.5 g) were excised into 6 vol (w/v) ice-cold homogenizing buffer (0.25 M sucrose; 10 mM TRIS-acetate, pH 7.8 at 20 C; 1 mM ethylene diamine tetraacetic acid (EDTA). Livers were coarsely chopped and homogenized and microsomes isolated by differential centrifugation (5). Half of the microsomal pellet was resuspended in 0.1 M TRIS-acetate (pH 8.0 at 20 C), 8 mM $CaCl_2$ (20 mg/ml) for subsequent assay; the other half was resuspended in extracting buffer (0.1 M TRIS-acetate, pH 8.0 at 20 C; 8 mM $CaCl_2$ and 0.1% Triton X-100; 2-3 mg/ml) for further purification. Purified, lipid-free microsomal phospholipase A_2 was obtained using techniques (8) that included Triton extraction, ammonium sulfate precipitation (35-65%) and gel filtration chromatography on Sephadex G-200.

Enzyme Assays

Phospholipase A_2 (PLA_2) activity was estimated by measuring the amount of radioactivity in intact phosphatidylcholine (PC), lysophosphatidylcholine (LPC) and free fatty acid following incubation of microsomes or aliquots of active column fractions with the radiolabeled substrate. The assay media were prepared by adding 0.1 μCi [^{14}C]PC and enough unlabeled PC to bring the concentration to the desired level in a test tube and evaporating the organic solvents under a stream of N_2. In assays of disaturated PC, the radiolabeled tracer, 1-palmitoyl,2-[^{14}C]stearyl PC, was supplemented with unlabeled dipalmitoyl PC. Otherwise, the radiotracer was identical to the unlabeled lipid. Following addition of assay buffer (0.1 M TRIS-acetate, pH 4.0 at 20 C, 8 mM $CaCl_2$, 0.1% Triton X-100), the mixture was sonicated for 2 min in a bath sonicator (Bransonic 12) and allowed to equilibrate for 5 min at either 5 C or 20 C. The assays were initiated by adding either 2 mg of microsomal suspension of aliquots of active column fractions at pH 8.0 to bring the final assay mixture to a volume of 1.0 ml and a pH of 6.5. The reaction was terminated with 3 ml of $CHCl_3$/MeOH (1:2, v/v) and lipids were extracted with organic solvents (12). The lipid products were separated by TLC into PC, LPC and free fatty acid fractions and counted using liquid scintillation methods, as previously described (5). Counts per min were converted to disintegrations per min using quench correction. Four concentrations were used of each substrate: 66.6 μM, 100.0 μM, 200.0 μM and 400.0 μM. Initial velocities were determined after 45 min, a duration that fell within the range of linearity of hydrolysis with time at all concentrations (data not shown). Kinetic parameters (Km and V) were estimated by weighted curve-fitting analyses of substrate concentration vs initial velocity (13), each velocity representing the mean from 2 experiments with 2 replicates per experiment.

Protein concentration was determined using the technique of protein-dye binding according to Bradford (14).

RESULTS

Phospholipase A_2 from liver tissue of rainbow trout is typical of the enzymes of lipid

metabolism in its ability to act on several different substrates. The preference of the enzyme for different molecular species was determined from kinetic data; substrates with the highest V/Km ratio being most preferred. The catalytic coefficient, C_{cat}, has been defined as the ratio of

$$\frac{V_1/Km_1}{V_2/Km_2}$$

(15), where V_1 and Km_1 are derived from the most "physiological" (or reference) substrate and V_2 and Km_2 are constants for the substrate of comparison. In this case, no a priori designation of a single most physiologically appropriate substrate exists, so all possible comparisons have been presented in Figure 1. Values of C_{cat} greater than unity indicate a preference of the enzyme for the species in the numerator.

Acclimation history of the fish had the greatest influence on C_{cat}, with enzyme from fish acclimated to cold temperatures exhibiting a clear preference for 1-palmitoyl, 2-oleoyl phosphatidylcholine (2-18:1 PC) followed by 1-palmitoyl,2-linoleoyl phosphatidylcholine (2-18:2 PC), then 1-palmitoyl,2-stearoyl phosphatidylcholine (2-18:0 PC). PLA_2 from fish acclimated to warm temperatures generally showed a preference for the disaturated species, 2-18:0 PC, but assay temperature influenced this substrate preference, particularly in the microsomal preparation, i.e., microsomes assayed at 20 C preferred 2-18:1 PC over both the disaturated and 2-18:2 PC, but at 5 C, 2-18:0 was the preferred substrate.

In its substrate preference, purified PLA_2 was similar to the particulate preparations with the exception of fish acclimated to warm temperatures that were assayed at 5 C, for which the particulate enzyme preferred the acyl moieties 18:0 > 18:2 > 18:1, but lipid-free enzyme preferred 18:2 > 18:0 > 18:1. At 20 C, particulate enzyme preferred 18:1 > 18:0 > 18:2 but purified enzyme preferred 18:0 > 18:2 > 18:1. Thus, both acute temperature change and the presence of endogenous lipids had a greater influence on the substrate preference of PLA_2 from fish acclimated to warm temperatures than cold.

Changes in the catalytic constant with acclimation history and assay temperature were a function of effects on both maximal velocity (V) and the Km (Table 1). The clear preference for 2-18:1 PC in PLA_2 from fish acclimated to cold temperatures was caused in most cases by

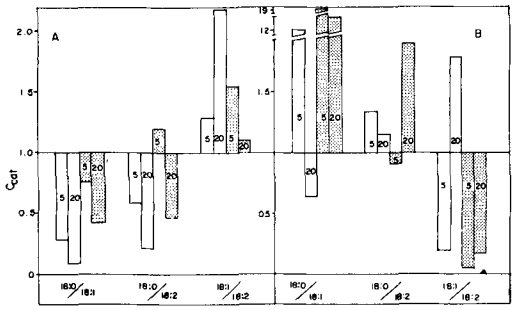

FIG. 1. Catalytic constant (C_{cat}) of microsomal PLA_2 from thermally acclimated rainbow trout. The activity of PLA_2 was measured at 4 different concentrations of 3 different PC substrates, 1-16:0, 2-18:0 PC, 1-16:0, 2-18:1 PC, and 1-16:0, 2-18:2 PC. C_{cat} is the ratio of kinetic constants,

$$\frac{V_1/Km_1}{V_2/Km_2}$$

for the different substrates taken 2 at a time. A ratio of 1.0 indicates no preference of the enzyme. Panel A is data from 5 C-acclimated trout and panel B is from 20 C-acclimated trout. Open bars are data from particulate, microsomal preparations and shaded bars are from purified, lipid-free preparations of PLA_2. Numbers in the bars represent the temperature at which the assay was performed.

low values of apparent Km. Maximal velocity increased with increasingly unsaturated substrates in microsomes of fish acclimated to cold temperatures, but the trend was not consistent in purified PLA_2 preparations. The enzyme from fish acclimated to 20 C, on the other hand, was most active (i.e., had the highest V/Km ratio) against the disaturated substrate at 20 C when in purified form, becaused of high V and low Km values. Thus, whereas assaying the microsomal PLA_2 in the absence of membrane lipids changed the relationship between individual kinetic parameters of the enzyme with different substrates, in most cases the overall substrate preference was the same as that in particulate enzyme because changes in Km were offset by changes in V.

For 2 of the 3 substrates, 2-18:1 PC and 2-18:2 PC, an increase in assay temperature resulted in higher V/Km ratios due to the acute effects of temperature which both increased V and decreased Km. With the disaturated substrate, however, reaction rates

TABLE 1

Kinetic Constants (± SEM) of Microsomal PLA_2 with Different Species of 1-Palmitoyl PC as Substrate

		Acclimated to 5 C			Acclimated to 20 C		
		18:0	18:1[b]	18:2	18:0	18:1[b]	18:2
Microsomes							
V[c]	5 C	4.81	12.18	62.92	11.11	21.02	23.14
		(0.78)	(0.28)	(10.38)	(1.91)	(1.35)	(2.26)
	20 C	84.04	34.27	22.86	2.74	94.21	138.26
		(15.9)	(2.37)	(2.03)	(0.19)	(6.44)	(25.4)
Km[d]	5 C	447	330	2174	148	1030	416
		(64)	(16)	(289)	(26)	(92)	(42)
	20 C	6005	222	337	111	2463	6471
		(307)	(25)	(36)	(17)	(157)	(321)
V/Km	5 C	0.011	0.037	0.029	0.075	0.020	0.056
	20 C	0.014	0.154	0.068	0.025	0.038	0.021
Purified							
V[e]	5 C	372	238	1316	1704	402	19280
		(25)	(12)	(223)	(151)	(7)	(5945)
	20 C	553	523	1523	1134	261	4167
		(109)	(36)	(206)	(107)	(11)	(708)
Km	5 C	385	188	1600	790	3465	7739
		(15)	(17)	(272)	(83)	(488)	(1234)
	20 C	658	261	828	145	406	1005
		(141)	(23)	(115)	(23)	(26)	(171)
V/Km	5 C	0.97	1.25	0.82	2.26	0.12	2.49
	20 C	0.84	2.00	1.84	7.83	0.65	4.17

[a]Liver microsomes and purified PLA_2 were assayed at 4 substrate concentrations and 2 acute temperatures. Numbers are means of 2 experiments. The symbols, 18:0, 18:1 and 18:2, represent the fatty acyl moieties at the *sn*-2 position of 1-palmitoyl PC substrates.

[b]Data for 1-palmitoyl,2-oleoyl PC has been reported previously (8).

[c]Units of V=nMol fatty acid released (mg hr)$^{-1}$.

[d]Units of Km = μm.

[e]V is reported as specific maximal velocity, therefore purified values are significantly higher than those for microsomes.

exhibited either no temperature sensitivity, or an inverse response of V/Km in every case except the purified enzyme from fish acclimated to warm temperatures. The enzyme from fish acclimated to 20 C had higher V values at 5 C than at 20 C in both preparations. In fish acclimated to cold temperatures, the lack of temperature sensitivity of V/Km was caused by increases in Km with temperature that offset the normal effects of acute temperature change on V.

DISCUSSION

Because enzymes typically operate at less than saturating substrate concentrations (below V), estimates of substrate preference derived from in vitro measurements, which include both V and Km, provide more insight into the in vivo catalytic rate. Analyses that include just one of these parameters may yield quite different results than those that include both (as is evident from the data here). The catalytic constant,

$$\frac{V_1/Km_1}{V_2/Km_2}$$

may be used to determine the preference of an enzyme for two substrates, 1 and 2, using the kinetic characteristics of each (15).

Using 3 molecular species of PC, we have demonstrated that the microsomal PLA_2 from the liver of rainbow trout exhibits substrate preferences consistent with the acyl substitution previously reported to occur (7) as a consequence of thermal acclimation. Hazel (7) reported that cold acclimation resulted in an increase in long-chain polyenoic acids, but a decrease in the proportions of both 18:1 and 18:2 at the *sn*-2 position of PC, from 12.40% at 20 C to 4.44% at 5C for 18:1 and 12.79%-5.22% for 18:2. The proportions of 18:0 were typically low regardless of acclimation condition, i.e., 0.61% at 5 C and 0.33% at 20 C. The

phospholipase A_2 from trout acclimated to cold temperatures showed an order of preference of 2-18:1 PC > 2-18:2 PC > 2-18:0 PC. In contrast to procaryotes (16), monoenes and dienes have not been shown to be important in maintaining membrane fluidity at low temperatures in fish. This selectivity of PLA_2 may account for the low levels of mono- and dienoic molecular species of PC at low temperatures. The low activity of PLA_2 toward disturated PC in fish acclimated to cold temperatures may reflect its infrequent occurrence as a substrate in vivo. In contrast, both membrane-bound and solubilized PLA_2 from trout acclimated to warm temperatures clearly preferred 2-18:0 PC > 2-18:2 PC > 2-18:1 PC when assayed at 5 C. Furthermore, the activity of PLA_2 toward disaturated PC in intact microsomes was inversely affected by acute temperature change (i.e., rates were higher at 5 C than at 20 C).

The transition temperature (T_c) of the disaturated species is 47 C, well above either of the assay temperatures employed. The species of PC with unsaturated fatty acids at carbon 2 have T_cs well below the cold assay temperature of 5 C (17). Thus, the disaturated PC was in a gel conformation at both assay temperatures, whereas the other PCs were presumably in a liquid-crystalline conformation. This may partially account for the lack of normal temperature sensitivity of PLA_2 when disaturated substrate was used, because the physical state of the substrate could influence the kinetic behavior of the enzyme.

The changes in substrate preference that accompanied thermal acclimation or changes in assay temperature could not be attributed to consistent effects on either of the kinetic parameters, V or Km. Comparisons between microsomal and lipid-free preparations, however, indicate that in most cases, the effects of acclimation on kinetic parameters were evident regardless of the presence or absence of endogenous membrane lipids, and, thus, were an attribute of the enzyme protein itself and not just the influence of the membrane lipids.

Studies of mammalian microsomal PLA_2 have indicated that the molecular species of preferred phospholipid substrates may be related to the tissue of origin of the enzyme in a physiologically meaningful way. Thus, in rat lung, dienoic species of PC were preferred substrates over monoenoic and disaturated species, which presumably served to enhance the accumulation of saturated PC for production of pulmonary surfactant (18). In bovine brain, the microsomal PLA_2 preferentially hydrolyzed saturated acyl moieties over polyenoic ones of phosphatidylinositol (PI), which accounted for the accumulation of arachidonate on PI, a form readily available for prostaglandin synthesis (19). Sevanian et al. (20) demonstrated a 2-fold enhancement of rat liver microsomal PLA_2 when peroxidized fatty acids occupied the *sn*-2 position of phospholipid substrates, implicating the enzyme in selective elimination of damaged acyl chains.

Consistent with these observations, the temperature-dependent substrate selectivity of trout microsomal PLA_2 indicates that phosphatide restructuring may be regulated at the deacylation step in the membrane metabolism of poikilotherms. The preference for disaturated PC by microsomal PLA_2 from trout acclimated to warm temperatures assayed at 5 C and the lack of temperature sensitivity in the hydrolysis of this species may contribute to the removal of saturated fatty acids from membrane phosphatides formed at high temperatures. The preference for the monoenoic PC by PLA_2 from trout acclimated to cold temperatures may provide a biochemical explanation for why polyenes but not monoenes accumulate in the membrane phospholipids of fish following cold acclimation. Further studies using longer chain polyenoic species of phosphatides are necessary to state this with certainty.

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REFERENCES

1. Cossins, A.R. (1981) in Effects of Low Temperatures on Biological Membranes (Morris, G.J., and Clarke, A. eds.) pp. 83-106, Academic Press, New York.
2. Dickens, B.F., and Thompson, G.A., Jr. (1982) Biochem is try 21, 3604-3611.
3. Hazel, J.R., and Neas, N.P. (1982) J. Comp. Physiol. 149, 11-18.
4. Holub, B.J., Piekarski, J., Cho, C.Y., and Slinger, S.J. (1976) J. Fish. Res. Board Can. 33, 2821-2826.
5. Neas, N.P., and Hazel, J.R. (1984a) J. Exp. Zool., submitted.
6. Jonas, R.E.E., and Bilinski, E. (1967) J. Fish. Res. Board Can. 24, 2555-2562.
7. Hazel, J.R. (1979) Am. J. Physiol. 236, R91-R101.
8. Neas, N.P., and Hazel, J.R. (1984b) J. Comp. Physiol., submitted.
9. Conner, A.M., Brimble, P.D., and Choy, P.C. (1981) Prep. Biochem. 11, 91-97.
10. Dickens, B.F., and Thompson, G.A., Jr. (1981) Biochim. Biophys. Acta 664, 211-218.
11. Rouser, G., Fleischer, S., and Yamamoto, A. (1970) Lipids 5, 494-496.
12. Bligh, E.G., and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911-917.
13. Wilkinson, G.N. (1961) Biochem. J. 80, 324-332.

14. Bradford, M.M. (1976) Anal. Biochem. 72, 248-254.
15. Mochizuki, Y. (1983) Comp. Biochem. Physiol. 74B, 299-302.
16. Sinensky, M. (1971) J. Bacteriol. 106, 449-455.
17. Chapman, D. (1975) Q. Rev. Biophys. 8, 185-235.
18. Longmore, W.J., Oldenborg, V., and van Golde, L.M.G. (1979) Biochim. Biophys. Acta 572, 452-460.
19. Gray, N.C.C., and Strickland, K.P. (1982) Lipids 17, 91-96.
20. Sevanian, A., Stein, R.A., and Mead, J.F. (1981) Lipids 16, 781-789.

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Double-Bond Patterns of Fatty Acids and Alcohols in Steer and Human Meibomian Gland Lipids

N. NICOLAIDES,* EVELYN C. SANTOS and **KONSTANTINE PAPADAKIS,** *Department of Medicine (Dermatology), The University of Southern California, School of Medicine, 2025 Zonal Ave., Los Angeles, CA 90033*

ABSTRACT

Ozonolysis studies of the monoenes of the fatty chain types in lipids of steer meibomian gland excreta (meibum) have confirmed earlier structural assignments based on gas liquid chromatography (GLC) retention data and have assisted in assigning complete structures to a group of recently identified ω-hydroxy fatty acids. The ω-hydroxy acids include straight-chain monoenoic acids (85%), saturated anteiso and iso acids (13%), monoenoic acids of the latter group (1%) and, finally, saturates of the normal monoenoic acids (1%). All the fatty chains of meibum can be biosynthesized by a unified process of chain buildup to primary chain lengths of 12:0-20:0 for the straight evens, with 16:0 predominating, 13:0-21:0 for the straight odds with 17:0 predominating, i16:0 to i28:0 for the iso and ai17:0 to ai29:0 for the anteiso chain types; then Δ9 desaturation of each of these chain types; and finally chain elongation of 1-10 C_2 units. Some chain degradation may also occur. The meibum lipid components involved are unsubstituted fatty acids, α-OH fatty acids, ω-OH fatty acids, fatty alcohols and some other lipid components incompletely characterized. The carbon skeletons are straight even, straight odd, iso and anteiso except that the α-OH fatty acids are only straight even and straight odd and these chains are not elongated. All fatty chains are almost entirely saturated and monoenoic, the polyenes occurring in only trace amounts. Biosynthesis of the fatty chains of human meibum evidently occurs similarly, except that considerably more 18:0 than 16:0 fatty acids are built up by the fatty acid synthetase, before desaturation and extension.
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INTRODUCTION

Within the eyelids of animals is a row of a sebaceous type of gland known as the meibomian gland that excretes lipid onto the edge of the eyelid. This lipid forms a surface layer on the tear film that probably retards loss of water by evaporation. It also provides a hydrophobic barrier preventing tear overflow and forms a water tight seal for the closed lids during sleep. It may have other functions.

In our efforts to define the lipid components of the meibomian gland excreta (meibum), we have recently (1) identified a group of ω-hydroxy fatty acids of unusually long chain lengths (C_{30} to C_{36}) that occurred to ca. 10% of the total acids of both steer and human meibum. Of the 3 homologous series reported, series 1 (85%) was monoenoic and series 2 (14%) was saturated but was not the saturated analog of series 1. This indicated that some additional structural difference existed between series 1 and 2. From gas chromatography (GC) retention data only, series 3 (1%) appeared to be the unsaturated analog of series 2, but this conclusion was not stated in the earlier work.

If the structural difference was methyl branching, as found in a number of natural fatty acids, clarification of questions as to the location of the double bonds and the methyl groups might be provided by ozonolysis studies. For example, if series 1 was unbranched, a reductive ozonolysis study of the acetylated methyl esters could locate the double bonds. If series 1 also possessed methyl branching on C-atoms different from those of the double bond, one could simultaneously identify the fragment with methyl branching, i.e., on either the aldehyde methyl ester (aldester) or the aldehyde acetate (aldacetate). Methyl branching on the C-atoms of the double bond would yield ketones.

To perform experiments of this type, standard aldesters and aldacetates of very long chain lengths would probably be required, especially if the double bond was close to either end. Some useful aldacetate standards might be obtained for this purpose from a reductive ozonolysis study of the unsaturated fatty alcohols (Falc) of meibum, and at the same time the double bond pattern of these alcohols could be established. This had not been done previously (2), and we knew that these unsaturated alcohols had long chain lengths, at least from C_{22} to C_{30}. To obtain potentially useful long chain aldester standards, confirming the double bond positional assignments of the unsubstituted fatty acid methyl esters (FAME) by ozonolysis studies and possibly extending these assignments to trace longer chain components seemed appropriate. We had earlier (2) made these assignments solely from retention data for fish FAME on free fatty acid phase (FFAP) glass capillary columns (3).

After determining the double bond positions of all the chain types found in meibum, we considered how all of these chain types could be formed by well-established biochemical processes. A relatively simple scheme that would account for all of them became apparent, and we report it here. To apply this scheme more fully, we also report here on the saturated structures of the substituted fatty acids of meibum, i.e., the α-hydroxy as well as the ω-hydroxy fatty acids, as the saturates of the other chain types have already been reported (2).

MATERIALS AND METHODS

A sample of total steer meibomian gland lipids (263.4 mg), previously purified by gel chromatography (Sephadex G-25) to remove nonlipid substances (4), was saponified by refluxing under an atmosphere of nitrogen with 20 ml of 10% KOH in 90% ethanol, w/v, for ca. 3 hr. The reaction mixture was then diluted with about the same volume of water, acidified with 6 N HCl and extracted 4 times with freshly redistilled chloroform. The extracts were pooled, counterwashed with water and dried to constant weight (253 mg).

To separate the saponifiables from the unsaponifiables, the extract was chromatographed on an alkaline SiO_2 column, 15.0 cm × 1.6 cm (Col. No. 1), as originally described (5) except that the SiO_2 adsorbent was Unisil (100-200 mesh, Clarkson Chemical Co., Inc., Williamsport, PA), and $CHCl_3/CH_3OH$ (95:5, v/v) was used to pack the column and elute the unsaponifiables (122.1 mg) instead of diethyl ether. Total fatty acids (135.6 mg) were eluted with $CHCl_3/CH_3OH/HCOOH$ (60:30:10). Methyl esters of total acids were prepared with 7% BF_3 in methanol (6), yielding 137.8 mg.

On a 9.0 cm × 1.6 cm SiO_2 column (Col. No. 2), to which 137.8 mg total FAME were applied, unsubstituted FAME (113.0 mg) were eluted with 10% benzene in hexane, α-hydroxy FAME (4.4 mg) with 50% benzene in hexane and ω-hydroxy FAME (13.6 mg) with $CHCl_3/CH_3OH$ (199:1). The α-hydroxy FAME (3.2 mg) were further purified by preparative thin layer chromatography (TLC) (2), yielding 0.9 mg and several unidentified components.

On a 17.0 cm × 1.0 cm column of $AgNO_3/SiO_2$, 7% w/w, (Col. No. 3) to which 45.1 mg unsubstituted FAME were applied, saturated FAME (24.0 mg) were eluted with hexane, monoenoic FAME (18.9 mg) with 20% benzene in hexane, dienoic FAME (1.7 mg) with 50% benzene in hexane and unidentified material (0.2 mg) with benzene. The adsorbent, previously dried at 115 C overnight, was deactivated with 15% water (w/w) and equilibrated for 3 hr before packing the column in hexane.

The unsaponifiables (122.1 mg) were chromatographed on a 20.0 cm × 2.8 cm SiO_2 column (Col. No. 4). This separated the wax alcohols (47.8 mg), eluted with 50% benzene in hexane, from sterols (51.2 mg) eluted with benzene, and from diols (9.0 mg) and other unidentified components (3.3 mg), eluted with 0.5% and 30% CH_3OH in $CHCl_3$. The wax alcohols were then acetylated (7), giving a yield of 49.6 mg. Saturated fatty alcohol acetates (37.9 mg) were separated from monoene acetates (4.1 mg) on a $AgNO_3/SiO_2$ column (Col. No. 5), as described above.

Analytical thin layer chromatography (TLC) was performed on 19-channel (20 cm × 20 cm) Whatman LK5D plates as in ref. 2. Solvents and other procedures for developing the TLC plates of column eluates were as follows: for Col. No. 1, $CHCl_3$/HAc (99:1) to 10 cm, followed by drying over a hot plate at 55 C for 5 min, then redeveloping with hexane/benzene (7:3) to the score line; for Col. No. 2, $CHCl_3$/benzene (4:6); and for Col. No. 4, earlier fractions with $CHCl_3$/hexane (9:1) and later fractions with $CHCl_3/CH_3OH$/conc. aq. NH_4OH (65:25:5) to an R_f of 0.3, drying on the hot plate as above, followed by $CHCl_3$ to the score line.

The monoenes of the different fatty chains were analyzed by preparative gas liquid chromatography (GLC), ozonolysis and analytical GLC. Hydrogenation (8) was done to check chain lengths. Preparative GLC was performed on a Varian 3700 instrument with a flame ionization detector (FID) and a micropacked stainless steel column (2.7 m × 1.02 mm i.d.) with 80-100 mesh Chromosorb WHP phase support and 3% SE-30 liquid phase. Ultrapure N_2 was the carrier gas, at an inlet pressure of 1.8 kg/cm^2, giving a flow rate of 5.4 cc/min at 100 C. Injector and detector temperatures were 350 C. Temperature programming was at 4°/min from ca. 140-315 C. The splitter sent 1 part of the effluent to the dector and 22 parts to collection tubes (straight glass tubes, 30 cm × 2 mm i.d.). We collected as often as necessary in the same tube to obtain sufficient material for further analysis. Collected material was transferred to glass stoppered centrifuge tubes with hexane and stored if necessary at −20 C before ozonolysis.

The procedure for reductive ozonolysis was modified from that of Stein and Nicolaides (9). An apparatus similar to that of Demole and Wuest (10) generated an estimated 0.9 mg of O_3/min from dry O_2 bubbling at the rate of 60 ml/min. The ozone/oxygen mixture was

bubbled for 1 min into a CS_2 solution of the compound to be ozonized at ca. −40 C. The excess O_3 and solvent was blown off immediately with N_2 at room temperature. The reduction of the ozonide was performed with a 2-fold molar excess of one of the following reductants: tri-*n*-butylphosphine, triphenylphosphine, tri-*p*-tolylphosphine, and tris-(*p*-methoxyphenyl)-phosphine (all from Aldrich Chemical Co., Milwaukee, WI), dissolved in the minimum quantity of CS_2 possible. A low volume of CS_2 was necessary to be able to see the very low molecular weight aldehydes that would otherwise be masked by the solvent in GLC. Thus, the C_6 aldehyde was completely separated from the narrow CS_2 solvent band when as much as 0.5 μl of solution was injected into the gas chromatograph. We found the choice of several reductants useful to avoid masking the aldester or aldacetate peaks with peaks from the excess of the particular reductant chosen and from its oxidation products.

Analytical GLC of samples and their ozonolysis products was performed on a Hewlett-Packard 5700A instrument with an FID, on a fused silica capillary column (30 m × 0.31 mm i.d.) chemically bonded with SE-30, 0.25 μm film thickness (J& W Scientific, Inc., Rancho Cordova, CA). The carrier gas was ultrapure N_2 at an inlet pressure of 0.9 kg/cm^2, giving a flow rate of 3 ml/min at ambient temperature; injector and detector temperatures were 350 C; the column was kept at 75 C for 2 min then programmed at 8°/min to 300 C. Equivalent chain length (ECL) values were obtained by isothermal glass capillary GLC on an FFAP column as previously described (2). Replicate values were easily within ±.04 ECL units.

Assignments of structures of FAME as normal, iso or anteiso were on the basis of precise comparison of retention times with standards and by further confirmation by GC-MS (mass spectrometry) as described in (2). Normal, iso and anteiso aldehydes formed by ozonolysis were identified as in ref. 11.

The percentage of each position isomer was calculated on the basis of its total counts of aldehyde plus aldester (or aldacetate) from the integration of the FID response with an Autolab Minigrator (Spectra Physics, Santa Clara, CA). This method is a practical approximation for w/w percentage in structures with a more centrally located double bond and avoids the necessity of calibration of the detector response separately to aldehydes and aldesters (or aldacetates) for determining position isomers of this type. Structures with a double bond near the ω end were invariably present in minute amounts.

RESULTS AND DISCUSSION

Ozonolysis studies of the fatty chain types known to occur in meibum have yielded 2 kinds of information. First, they have shed some light on the nature of the structural differences between the various series of the newly identified ω-hydroxy fatty acids described in the Introduction and listed in Table 1. Second, they have led to the recognition of a unified scheme of how the different fatty chain types of the various lipid components could be biosynthesized.

Because ozonolysis of the components of series 1 yielded only straight chain fragments, i.e., aldesters and aldacetates, this series must be straight chain and monoenoic. Even as well as odd members are present. The aldester and aldacetate fragments formed from ozonolysis were identified as straight chain from fragments with identical retention times obtained from straight chain monoenoic FAME and Falc acetates.

The structural feature of series 2 is likely to be methyl branching of the iso and anteiso type, as is true of all the other branched chain compounds found in meibum. We believe this

TABLE 1

ω-Hydroxy Fatty Acids of Steer Meibum[a]

	Series 1[b] monoenes			Series 4 saturated		
	Normal	Even (%)	Odd (%)	Normal	Even (%)	Odd (%)
C_{30}		6.2			0.5	
C_{31}			6.1			tr
C_{32}		39.1			0.4	
C_{33}			7.7			tr
C_{34}		23.6			tr	
C_{35}			0.3			tr
C_{36}		1.6			tr	
C_{38}		tr				
		70.5	14.1		0.9	

	Series 2[b] saturated		Series 3[b] monoenes	
	Iso (%)	Anteiso (%)	Iso (%)	Anteiso (%)
aiC_{29}		1.0		
iC_{30}	0.7			
aiC_{31}		5.8		0.1
iC_{32}	1.2		0.3	
aiC_{33}		4.8		0.4
iC_{34}	tr		0.2	
aiC_{35}		tr		
	1.9	11.6	0.5	0.5

[a]As the acetate or TMS derivative of the fatty acid methyl ester. They constitute 9.9% of the total acids.
[b]Series 1, 2 and 3 were identified in ref. 1.

to be true because series 3, the unsaturated analog of series 2, gave aldacetates with some structural feature, presumed here to be methyl branching, but aldesters that were straight for the 2 isomers ozonized (Table 2). This is what one would expect for branching of the iso or anteiso type. Furthermore, the ECL values for the branched C_{30}, C_{31}, C_{32} and C_{33} chains were 29.42, 30.42, 31.38 and 32.42 when the hydrogenated total sample of the acetoxy derivatives of all the ω-OH FAME were gas chromatographed isothermally on Dexsil. These values are entirely reasonable if one considers that the acetoxy group extends the chain by what amounts to ca. 3 C-atoms. This would reduce the fractional chain length (FCL) values, normally at ca. 0.60 for iso and 0.72 for anteiso, to ca. 0.42 found for more centrally branched methyl group positions (12,13). The same applies to the aldacetates, which also gave FCL of ca. 0.40 on ozonolysis of series 3. We are, therefore, designating the even chain branching as iso and the odd chain branching as anteiso, and the fact that anteiso is the favored branch chain type for other compounds of steer meibum, as it is for the ω-OH FAME, is consistent with the correctness of this assumption. NMR data on these fractions, to be published, also support this conclusion. Thus, in summary, detailed examination of the ω-OH fatty acids has shown that they are a complex group of exceedingly long-chain substances. The major fraction, earlier called series 1, is straight chain and unsaturated with odd and even members present. An appreciable fraction, earlier called series 2, is saturated, the bulk of which are very likely anteiso analogs, with small amounts of iso analogs also present. The fraction earlier called series 3 consists of most probably iso and anteiso unsaturated analogs. Series 4, not reported earlier, which occurs in trace amounts, are saturated normal ω-hydroxy fatty acids (Table 1).

Meibum releases mainly 4 types of aliphatic lipid components on saponification. These are unsubstituted fatty acids, fatty alcohols, α-hydroxy fatty acids and ω-hydroxy fatty acids. These lipid components occur in primarily 4 different carbon skeletal forms: straight even, straight odd, iso even and anteiso odd. These are the same aliphatic chain types that occur in many kinds of sebaceous products (14,15). Most compounds of these chain types occur as saturates or as monoenes. Very few dienes and only traces of trienes occur, as has been found for meibum in the present study. In work to be reported, we have also found evidence for the occurrence of small amounts of α,ω diols. A very small amount of iso odd chains occur as fatty acids and fatty alcohols. We have not been able to determine their double-bond patterns because they emerged from the gas chromatograph too close to the relatively large amounts of anteiso odd chains.

TABLE 2

Double-Bond Patterns of Acetates of ω Hydroxy Normal and Branched Monoenoic Fatty Acids (as Methyl Esters) of Steer Meibum

	30:1 (%)	31:1 (%)	32:1 (%)	33:1 (%)	34:1 (%)	35:1 (%)	36:1[a] (%)	Total ω pattern (%)
Pattern								
ω7	87	→	49	→	18	→	22	→ 34.1
ω9	13	→	51	→	82	→	78	→ 48.3
ω8		97	→	93	→	100		15.7
ω10		3	→	7				0.8
								98.9
Percentage of all monoenoic ω-OH FAME	7.2	7.1	45.9	9.0	27.4	0.4	1.9[b]	Σ = 98.9

	ai31:1 (%)	i32:1 (%)	ai33:1 (%)	i34:1 (%)	
Pattern					
ω9		74	→	64	0.4
ω11		26	→	36	0.2
					0.6
Percentage of all monoenoic ω-OH FAME	0.1	0.4	0.5	0.2[b]	Σ = 1.2

[a]A trace of what appeared to be 38:1 was seen but the double-bond position could not be determined.
[b]Percentages reported here are based on 100% monoenes. Of total ω-OH fatty acids, ca 86% were monoenes and ca 14% saturates.

Monoenes of the lipid components of this study can be efficiently discussed in terms of ω patterns. We define an ω pattern as a sequential group of monoenes where the double bond of each member occurs on the same C-atom numbering from the ω end of the chain. Such patterns suggest similar modes of biosynthesis as each member differs from the others by an integral number of $-CH_2-CH_2-$ units (C_2 units). In Table 3, for example, 5 ω patterns are found of straight even FAME (ω3-ω11) and 5 ω patterns of straight odd FAME (ω4-ω12). The total amount of each ω pattern can be calculated by summing the products obtained by multiplying the percentage of that ω isomer for each chain length by the percentage of that chain length of the total monoenes, which is listed at the bottom of Table 3. For example, to calculate the total ω7 pattern, one would add the product of 0.18 × 1.5% for 14:1 plus 0.77 × 27.1% for 16:1 plus 0.40 × 48.6% for 18:1 plus the corresponding products for all the other chains up to 32:1, which would be 45.1%, as listed in the last column of Table 3.

For the straight even monoenes of the FAME, we can account for the synthesis of all the homologs according to 4 well-established biochemical processes: (a) starting with acetyl-CoA, a fatty acid synthetase builds up the straight even chains from 12:0 to 20:0, which we are calling the primary saturated chain lengths, then (b) a Δ9 desaturase removes an H-atom from the 9th and the 10th C-atoms in apparently only these primary saturated chains (indicated by a diagonal dotted line in Table 3), (c) the resulting monoenes are then extended to other chain lengths, up to a maximum of 34:1 or 10 C_2 units, as observed by us and (d) some degradation may also occur (indicated by dotted arrows to the left of the diagnal dotted line in Table 3), but we have no evidence for this. We would not be aware of any chain degradation occurring to the right of the diagonal line. Note that the diagnal dotted line would have to be extended to 12:1 to account for the trace amount of the ω3 pattern seen at 16:1 (see footnote c, Table 3). Note also that any ω3 for 14:1 was too small to be detected by our methods.

A similar scheme would explain all of the monoenes of the straight odd FAME: (a) starting with propionyl-CoA, the fatty acid synthetase builds up the primary straight odd chain saturates from 13:0 to 21:0, then (b) a Δ9 desaturase removes 2 H-atoms from apparently only these chain lengths, (c) the monoenes so formed are further extended to other chain lengths, up to a maximum of 33:1 as observed by us and (d) as with the straight even chains, a small amount of chain degradation may occur. Again the diagonal dotted line would have to be extended, this time to 13:1 for the same Δ9 desaturase to account for the small amounts of the ω4 pattern for 15:1, 17:1, 19:1 and 29:1. We must also assume that the missing intermediates to form 29:1 were present in amounts too small to be detected by our methods.

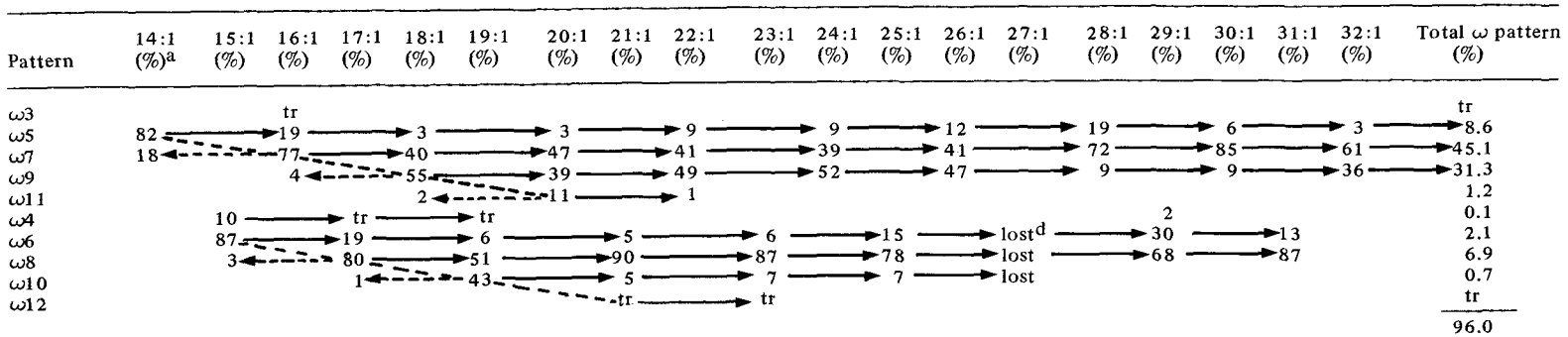
The entire chain-building processes for the straight even chains and the straight odd chains are strikingly similar in that (a) the straight odds have the same number of ω patterns as the straight evens, (b) only one desaturase, which removes 2 H-atoms at Δ9, is required to account for all the monoenes present, and (c) these initially desaturated chains are further extended to almost the same remarkably long lengths.

In the iso and anteiso chains (Table 4), a similar scheme could explain all of these monoenes: (a) starting with isobutyl-CoA or with 2-methylbutyl-CoA, the fatty acid synthetase builds up, respectively, iso even chains from i16:0 to i28:0 and anteiso odd chains from ai17:0 to ai29:0, then (b) a Δ9 desaturase removes 2 H-atoms from apparently only these chain lengths and (c) the monoenes so formed are further extended to other chain lengths, up to a maximum of i30:1, and ai31:1. Again, the dotted line should be extended to i16:1 and ai17:1 and intermediates from i20:1 to i26:1 and from ai21:1 to ai27:1 were too small to be detected and (d) again some degradation may occur for the anteiso chains.

The buildup of all the monoenoic fatty alcohols—straight even, straight odd, iso even and anteiso odd—is strikingly similar to that of the monoenoic fatty acids (Tables 5 and 6). The fatty acid synthetase first forms the primary chain lengths of each skeletal type, i.e., 12:0 to 20:0 for the straight even chains, 13:0 to 21:0 for the straight odd chains, i16:0 to i28:0 for iso chains and ai17:0 to ai29:0 for anteiso chains. The Δ9 desaturase acts on each of these forming monoenes, and finally the elongase extends these, to a maximum of 34:1 for the ω5, ω7 and ω9 patterns of the straight even chains, to 33:1 for the ω6, ω8 and ω10 patterns of the straight odd chains, to i34:1 for the ω9 and ω11 patterns of the iso even chains, and to ai33:1 for the ω8, ω10, ω12, ω14 and ω16 patterns of the anteiso odd chains (Tables 5 and 6). Again, the major patterns are the ω7 and ω8 for the even and odd chains (Table 5), suggesting that, as with the fatty acids, the primary products of the synthetase are 16:0 and 17:0. However, with the alcohols, no saturated equivalents of the unextended chains are found, as is the case with the fatty

TABLE 3

Double-Bond Patterns of Normal Even add Odd Monoenoic Fatty Acids (as Methyl Esters) of Steer Meibum



[a]Percentage of each isomer was obtained by ozonolysis. When a C_4,C_5 or branched aldehyde fragment was masked by solvent, assignment of double-bond position was made on the basis of the aldester or aldacetate fragment only.

[b]Solid arrows indicate possible C_2 biosynthetic addition. Dotted arrows indicate possible C_2 biosynthetic degradation. Items on the diagonal dotted line are suggested points of introduction of double bonds.

[c]12:1 (0.2%), 33:1 (tr) and 34:1 (tr) were detected but not included in table as corresponding ω values were not obtained.

[d]Value for 27:1 is not included in computation of total ω pattern becaused sample was lost.

[e]The difference between 100% and this value is the sum of the ωpattern of the iso and anteiso monoenes (Table 4) plus that accounted for by footnote c.

TABLE 4

Double-Bond Patterns of Monoenoic Iso and Anteiso Fatty Acids (as Methyl Esters) of Steer Meibum[a]

Pattern	i18:1[b] (%)	ai19:1[b] (%)	i20:1 (%)	ai21:1 (%)	i22:1 (%)	ai23:1 (%)	i24:1 (%)	ai25:1 (%)	i26:1 (%)	ai27:1 (%)	i28:1 (%)	ai29:1 (%)	Total ω pattern (%)
ω7	29												
ω9	71	→ 4	→ 71	→ 20	→ 14	→ 2							0.1
ω11		96 →	16 →	20 →	23 →	12							0.4
ω13			67 →	15 →	3 →	25							0.8
ω15				45 →	44 →	5							0.2
ω17					16 →	11							0.4
ω19						32							0.1
ω8		tr				13							tr
ω10		93 →	7 →	9 →	11 →	8 →	6						tr
ω12		4 ←	93 →	13 →	12 →	7 →	13						0.2
ω14		3 ←		78 →	30 →	5 →	9						0.4
ω16					47 →	71 →	11						0.3
ω18						9 →	19						0.6
ω20							30						0.1
							12						0.1
													3.6
Percentage of all unsubstituted monoenoic FAME[c]	0.3	0.1	0.5	0.3	0.2	0.2	0.3	0.5	0.6	0.4	0.1	0.1	Σ = 3.6

[a]Refer to applicable footnotes in Table 1.

[b]i refers to iso branching and ai refers to anteiso branching.

[c]ai15:1 (tr), ai17:1 (0.1%), i30:1 (tr) and ai31:1 (tr) were detected but not included as corresponding ω values were not obtained.

acids. A similar situation exists for the branched Falc monoenes (Table 6), which make up less than 6% of the total monoenoic alcohols. The close similarity of the structures of the alcohols to the structures of the extended fatty acids is also notable, in an additional sense, for in another type of sebaceous gland, the uropygial glands of many birds, the alcohols are normal, whereas the acids are polybranched (16).

About half of the α-OH FAME (Table 7) are unsaturated. Again, Δ9 desaturation occurs on the primary chains built up by the fatty acid synthetase with 16:1 predominating. The extensive chain elongation found for the FAME and the Falc does not occur with this lipid component and only straight odd and straight even chains occur.

The ω-OH FAME, on the other hand, show the same processes that occur for the FAME and the Falc but with certain distinct features (Table 2). For these substances, 16:0, 17:0, 18:0, 19:0, i18:0, i20:0 and possibly ai19:0 and ai21:0 appear to be the chains that are first built up by the fatty acid synthetase, then Δ9 desaturation occurs followed by chain elongation, the longest chain being 38:1. Finally, the long chains are hydroxylated at the methyl end. Hydroxylation occurs to a small extent even on some iso and anteiso chains. Besides ω-hydroxylation, hydroxylation on the anteiso methyl carbon could possibly occur. If it did, it would yield an α-branched ethyl group. We did not determine the double-bond positional isomers of these anteiso chains, because of the very small amounts present, and because the large amounts of saturated anteiso homologs masked them in preparative GC.

For the normal ω-OH acids, the proportion of isomers of the ω9 pattern is, in contrast to the other normal monoenes, somewhat greater than for the ω7 pattern (Table 2). As with the alcohols, the ω-OH fatty acids have no unelongated chains and the elongations are remarkably long.

All ω patterns of all chain types are summarized in Table 8. Considering the relative amounts of the various straight even ω patterns, substantially more acetyl-CoA must be available than propionyl-CoA available for initial chain buildup of primary chains. Also, note that within the straight even chains, ω7 > ω9 > ω5 > ω11 > ω3. This distribution parallels the distribution of the amounts of the primary straight even saturated FAME (also listed in Table 8) from which each of these ω patterns could be formed by Δ9 desaturation, i.e., 16:0 > 18:0 > 14:0 > 20:0 > 12:0. The fact that the amount of each primary saturated chain remaining at that chain length parallels

TABLE 5

Double-Bond Patterns of Normal Even and Odd Monoenoic Fatty Alcohols (as Acetates)

Pattern	22:1 (%)	24:1 (%)	25:1 (%)	26:1 (%)	27:1 (%)	28:1 (%)	29:1 (%)	30:1 (%)	31:1 (%)	32:1 (%)	33:1 (%)	34:1 (%)	Total ω pattern (%)
ω3		tr											tr
ω5	4	16		41		20		6		–		4	12.0
ω7	57	46		28		67		63		39		32	45.8
ω9	39	38		31		13		29		55		64	22.8
ω11								2		6			1.2
ω4			8		22		2						0.6
ω6			17		43		22		11		2		2.5
ω8			71		33		72		86		91		8.7
ω10			4		2		4		3		7		0.4
													94.0
Percentage of all monoenoic fatty alcohols[b]	0.1	2.7	1.0	11.0	2.0	25.1	4.6	33.0	4.2	9.4	0.4	0.5	Σ = 94.0

[a]Refer to applicable footnotes in Table 1.
[b]23:1 (0.1%) was not included as its ω values were not obtained.

TABLE 6

Double-Bond Patterns of Monoenoic Iso and Anteiso Fatty Alcohols (as Acetates) of Steer Meibum[a]

Pattern	ai23:1 (%)	i24:1 (%)	ai25:1 (%)	i26:1 (%)	ai27:1 (%)	i28:1 (%)	ai29:1 (%)	i30:1 (%)	ai31:1 (%)	i32:1 (%)	ai33:1 (%)	i34:1 (%)	Total ω pattern (%)
ω7				1	3	3							tr
ω9		53	48	37	50	62	89						1.4
ω11		37	35	40	44	18	11						1.0
ω13		6	5	5	1	17							0.2
ω15		4	11	4	1	3							0.2
ω17				10	tr	tr							0.1
ω19				tr	tr								tr
ω4	15												tr
ω6	7	4											tr
ω8	75	7	5	18	20	9							0.4
ω10	3	44	40	54	44	33							1.2
ω12		28	24	17	29	15							0.7
ω14		13	7	4	5	40							0.2
ω16		4	24	2	1	3							0.3
ω18				5	1								tr
													5.7
Percentage of all Monoenoic fatty alcohols[b]	tr	0.1	0.2	0.9	0.9	0.7	0.7	0.8	0.8	0.4	0.2	tr	Σ = 5.7

[a]Refer to applicable footnotes in Table 1 and 2.

[b]ai21:1 (tr), i22:1 (0.2%), ai23:1 (tr) and i24:1 (tr) were not included as their ω values were not obtained.

TABLE 7

α-Hydroxy Fatty Acids (as Methyl Esters) of Steer Meibum[a]

α-OH acid	Saturates (%)	Unsaturates (%)
14:0	0.2	
14:1		0.1
15:0	4.9	
15:1ω6		1.5
16:0	46.9	
16:1ω5		3.0
16:1ω7		34.7
17:0	0.2	
17:1ω8		6.4
18:0	0.2	
18:1ω9		1.8
	52.4	47.5

[a]α-OH FAME = 0.9% of total FAME of steer meibum.

the amount of that saturate used to synthesize the components in a given ω pattern suggests that the Δ9 desaturase selects chains for desaturation randomly, at least for the straight even chains. In the case of straight odd chains, the distribution is ω8 > ω6 > ω10 > ω4 > ω12, which does not exactly parallel the distribution of the amount of corresponding saturated substrates for random monoene formation of this chain type, i.e., 17:0 is not greater than 15:0 as would be predicted by a random process whereby 17:0 > 15:0 > 19:0 > 13:0 > 21:0 (Table 8). The only likely explanation we have for this difference is that the amounts in question are very small, making the error of measurement more significant.

The ω6 pattern is structurally analogous to that of essential fatty acids. This pattern is built up by the steer, not by a plant. Plants are commonly supposed to be the sole source of these acids. However, the ω6 pattern in this case produces an odd chain length rather than the even one normally considered to be "essential." Furthermore, these acids are produced for external uses by holocrine excretion and are not available for any further internal use.

The iso and anteiso monoenoic FAME comprise 2.0% and 1.6% of the total ω patterns in contrast to the straight evens and straight odds that make up 86.2% and 9.8% (Table 8). Considering the fact that all the monoenes make up ca. 40% of the total unsubstituted acids in meibum, of the primary saturated chains built up, excluding those forming fatty alcohols, ca. 80% of the straight evens and 80% of the straight odds were desaturated. In contrast, 11% of the primary saturated iso chains and only 3% of the anteiso chains were desaturated, again excluding those forming alcohols. This implies that the methyl branch of these chains impedes the desaturation process, and that the anteiso branch impedes it more than the iso branch, especially for the lower ω patterns, i.e., those closer to the ω end. Thus, we confirm the observation of Brett et al. (17), who found considerably less Δ9 desaturation for the iso and anteiso C_{18} acids when compared with stearic acid. However, although only very small amounts of unsaturates of iso and anteiso chains are formed, they constitute a large number of ω patterns, 7 each for iso and anteiso. The amounts of these ω patterns did not correlate significantly with the general distribution of primary saturated chains (Table 8).

Minor variations of the 4-step scheme postulated above, e.g., chain elongation or chain degradation preceding desaturation are also possible and, in some cases, may provide a better quantitative correlation of the amount of ω pattern with the amount of primary chain build up. The scheme proposed here for the formation of all the monoenes could, if the desaturation step is omitted, also account for all the saturates of the different chains occurring in meibum. Thus, in summary, study of the double-bond patterns of the various chain types occurring in steer meibum sheds a good deal of light as to how all the chains could be formed.

For the bulk of the FAME of steer and human meibum, our earlier assignments of ω values for indicating the location of the double bonds of a fatty chain, based solely on ECL obtained on FFAP glass capillary columns (2), were correct, i.e., for each chain length of 16:1, 18:1, 20:1, and 22:1, 3 position isomers were found: ω5, ω7 and ω9, with an additional isomer, ω11, for 20:1. For 14:1, the assignments of the ω5 and ω7 structures were also correct, but we cannot now account for the assignment of ω6, which occurred to the extent of .01% for this chain length. For 24:1 and 26:1, the assignments ω9 and ω11 structure were made in place of the correct ω7 and ω9 structures, and finally, for the odd chain lengths 15:1, 17:1, 19:1, 21:1 and 25:1, the assignment ω7 or ω9 was made in place of the correct ω8 structure. These misassignments were made largely on components occurring in minute amounts, which produced poorly shaped peaks, resulting in retention data of less than the accuracy required to make these assignments. These misassignments concealed the remarkably consistent ω patterns that have become manifest in the present study.

Although we did not perform ozonolysis of individual monoenes of human meibum, we did ozonize a sample of the entire unsubstituted

TABLE 8

ω Patterns of the Chain Types the Occur in Steer Meibum and Other Data

Type	ω Patterns				Saturated fatty acids (unsubstituted) needed for Δ9 desaturation	
	Fatty acid (unsubstituted) (%)	Fatty alcohols (%)	α-OH FA (%)	ω-OH FA (%)	Chain length	Percentage[a]
Normal even						
ω3	tr	tr			C_{12}	0.04
ω5	8.6	12.0	6.3		C_{14}	0.51
ω7	45.1	45.8	72.8	34.1	C_{16}	6.30
ω9	31.3	22.8	3.9	48.3	C_{18}	1.97
ω11	1.2	1.2			C_{20}	0.12
	86.2	81.8	83.0	82.4		8.94
Normal odd						
ω4	0.1	0.6			C_{13}	0.01
ω6	2.1	2.5	3.2		C_{15}	0.61
ω8	6.9	8.7	13.6	15.7	C_{17}	0.29
ω10	0.7	0.4		0.8	C_{19}	0.03
ω12	tr				C_{21}	tr
	9.8	12.2	16.8	16.5		0.94
Iso						
ω7	0.1	tr			C_{16}	0.84
ω9	0.4	1.4		0.4	C_{18}	0.50
ω11	0.8	1.0		0.2	C_{20}	0.61
ω13	0.2	0.2			C_{22}	0.39
ω15	0.4	0.2			C_{24}	1.59
ω17	0.1	0.1			C_{26}	2.15
ω19	tr	tr			C_{28}	0.32
	2.0	2.9		0.6		6.40
Anteiso						
ω4		tr				
ω6		tr				
ω8	tr	0.4			C_{17}	3.61
ω10	0.2	1.2			C_{19}	1.22
ω12	0.4	0.7			C_{21}	2.18
ω14	0.3	0.2			C_{23}	2.38
ω16	0.6	0.3			C_{25}	9.44
ω18	0.1	tr			C_{27}	4.80
ω20	tr				C_{29}	1.25
	1.6	2.8				24.88
Grand Totals	99.6	99.7	99.8	99.5		

[a]From Ref. 2.

FAME of human meibum and compared the products with a similar sample from the steer. The aldehydes released by this procedure are indicative of the relative amounts of the ω patterns, but are not quantitatively equivalent to them, because they do not take into account the amount of aldester (or aldacetate) in the various chains, which also contribute to the amount of ω pattern. In this fashion, we established that human meibum FAME are primarily ω7 and ω9 that, we assume, are derived from Δ9 desaturation of 16:0 and 18:0. This confirms the correct assignments of ω7 and ω9 based on ECL values in our earlier study (2) (Tables 9 and 10). Also included are small amounts of ω5, as in the steer.

Human meibum has ca. 2 times as much ω9 as ω7, which is in contrast to the steer FAME where the ω7 is ca. 1.5 times larger than the ω9 (Table 10). (Note also that the total ω patterns from this study and the earlier study for all the fatty chains tabulated are in close agreement.) This would mean that in the steer, the Δ9 desaturation of the FAME occurs mainly on a 16:0 acid, whereas in the human it occurs mainly on a 18:0 acid. For the odd chains, ω8 is predominant, indicating that, for both the steer and the human, Δ9 desaturation is mainly on 17:0, followed by chain extension. A small amount of ω6 (and probably ω10, not

TABLE 9

Equivalent Chain Lengths (ECL) of Monoenoic Fatty Acids and Fatty Alcohols of Steer and Human Meibum ECL of Fatty Acids as Methyl Esters

	Straight even									Straight odd					
	ω3	ω5		ω7		ω9		ω11		ω4	ω6		ω8		ω10
Fatty acid	This study	This study	Ref. 2	This study	Ref. 2	This study	Ref. 2	This study	Ref. 2	This study	This study	Ref. 2	This stydy	Ref. 2	This study
14:1	–	14.38	14.43	14.27	14.27	–	–	–	–						
15:1										15.46	15.32	15.36	15.24	15.25	–
16:1	16.57	16.39	16.43	16.28	16.31	16.22	16.23	–	–						
17:1										17.49	17.35	17.36	17.25	17.27	–
18:1	–	18.42	18.42	18.29	18.32	18.23	18.25	–	–						
19:1										19.48	19.38	19.36	19.27	19.26	19.21
20:1	–	20.43	20.45	20.30	20.36	20.23	20.30	20.16	20.27						
21:1										–	21.33	–	21.24	21.31	21.15
22:1	–	22.45	22.45	22.33	22.36	22.24	22.30	22.17	–						
23:1										–	23.41	23.36	23.28	23.30	23.22
24:1	–	24.49	24.45	24.34	24.32	24.25	24.24	–	–						
25:1										–	25.37	–	25.28	25.30	25.22
26:1	–	26.51	–	26.37	26.32	26.27	26.20	–	–						
27:1										–	–	–	–	–	–
28:1	–	28.47	–	28.35	–	28.23	–	–	–						
29:1										–	–	–	–	–	–
30:1	–	30.43	–	30.31	–	30.22	–	–	–						
						ECL of Fatty Alcohols as Acetates[b]									
22:1	–	22.47	–	22.34	22.33	22.26	22.24								
23:1										–	–	–	–	23.30	–
24:1	24.64	24.47	24.48	24.31	24.32	24.22	24.22								
25:1										25.56	25.40	–	25.30	25.27	25.16
26:1		26.48	26.48	26.34	26.32	26.24	26.22								
27:1										27.56	27.42	–	27.29	27.31	27.21
28:1	–	28.42	28.48	28.29	28.32	28.19	28.23								
29:1										29.55	29.40	–	29.29	29.30	29.18
30:1	–	30.44	–	30.33	30.33	30.20	30.22								
31:1										–	–	–	–	–	31.16

[a]ECL values listed under the heading "This study" were determined by capillary GC on FFAP columns of collected GC fractions solely from stear meibum, whose structure assignments are based on the present ozonolysis studies. ECL values listed under "Ref. 2" were also done by capillary GC on identical FFAP columns used for analysis of total samples of FAME and Falc acetates from steer and human meibum. ECL values used for the assignment of structures for straight even and odd monoenes were mostly within ±0.04 ECL units of the corresponding human monoenes. This made it possible to postulate probable structures of these human monoenes without having to do ozonolysis studies. Assignments of ω values in Ref. 2 deviating from the correct ones are discussed in the text.

[b]Assignment of ω values for the ECL listed are based on the present ozonolysis studies. They were not reported in Ref. 2.

TABLE 10

ω Patterns of FAME and Falc Acetates of Steer and Human Meibum

	Steer FAME (%)[a]										Human FAME (%)[a]				
	Straight even						Straight odd				Straight even			Straight odd	
	This study			Ref. 2			This study		Ref. 2		Ref. 2			Ref. 2	
Fatty acid	ω5	ω7	ω9	ω5	ω7	ω9	ω6	ω8	ω6	ω8	ω5	ω7	ω9	ω6	ω8
14:1	0.52	0.11	–	0.54	tr	–					0.10	0.14	–		
15:1							0.26	0.01	0.25	45				0.02	0.07
16:1	2.17	8.81	0.46	1.70	7.82	0.11					0.10	4.86	1.06		
17:1							0.52	2.19	0.58	1.94				0.03	0.73
18:1	0.62	8.20	11.28	0.87	7.73	10.37					0.26	6.34	24.63		
19:1							0.04	0.30	45	0.35				tr	0.07
20:1	0.02	0.36	0.30	0.05	0.53	0.41					0.06	0.97	0.43		
21:1							tr	0.08	–	0.12				–	tr
22:1	0.05	0.21	0.25	tr	0.36	0.33					0.07	0.70	0.36		
23:1							0.01	0.11	0.20	0.23				–	–
24:1	0.10	0.43	0.57	0.20	0.82	0.79					0.15	1.32	0.96		
25:1							0.03	0.16	–	0.74				–	–
26:1	0.07	0.22	0.26	–	0.35	0.31					–	0.87	0.49	–	–
	3.55	18.34	13.12	3.36	17.61	12.32	0.86	2.85	1.03	3.38	0.64	15.20	27.93	0.05	0.87
	Steer Falc Acetates (%)[a]										Human Falcs Acetates (%)[a]				
22:1	tr	0.01	tr	–	0.03	0.03					–	0.29	0.05		
23:1							–	–	–	tr				–	tr
24:1	0.04	0.12	0.10	0.10	0.29	0.18					0.34	2.70	1.93		
25:1							0.02	0.07	–	0.18				–	0.16
26:1	0.44	0.30	0.33	0.55	0.43	0.45					0.42	3.28	2.65		
27:1							0.08	0.06	–	0.14				–	–
28:1	0.49	1.63	0.32	0.70	1.68	0.34					0.28	3.80	0.84		
29:1							0.10	0.32	–	0.45				–	–
30:1	0.19	2.02	0.93	–	2.37	0.78					–	4.54	1.42		
	1.16	4.08	1.68	1.35	4.80	1.78	0.20	0.45	0	0.77	1.04	14.61	6.89	0	0.16

[a]The amounts of each position isomer of the steer and human monoenoic FAME and Falc acetates were computed as in Ref. 2 on the basis of the total unsubstituted FAME or Falc acetates (saturates and unsaturates included).

listed in Table 10) also occurs. Although we did not ozonize the fatty alcohols for the human, we can ascribe correct structures from their ECL values obtained in the present study compared with those of the earlier study (2) (Table 9). In this case, note that the human alcohols, besides having ca. 3 times as much unsaturation as the steer alcohols, also have more than twice the amount of $\omega 7$ as $\omega 9$ when compared with the steer. α-Hydroxy acids and ω-hydroxy acids also occur in the human, probably by similar mechanisms to those of the steer. Thus, we may assume that the biosynthesis of the fatty chains of human meibum parallels that of the steer meibum and that the monoenes are based on $\Delta 9$ desaturation of the same primary chains built up by the fatty acid synthetase (but more of 18:0 than 16:0 for the fatty acids) and that these chains are then extended again to the same remarkably long chain lengths as in the steer.

As was pointed out earlier (2), the unsaturates of human sebum are based on $\Delta 6$ desaturation of the same 4 skeletal types of primary chains built up by meibum. The sebaceous gland, however, excretes onto a dry skin surface, whereas the meibomian gland excretes on to a wet surface. Can this shift of ω patterns of unsaturation, based on $\Delta 6$ to those based on $\Delta 9$, have any functional advantage in the aqueous environment of tears? We have seen a similar shift in some of the unsaturates of vernix caseosa wax esters and sterol esters from $\Delta 6$ to $\Delta 9$ if one compares the ω patterns of adult sebum, excreted to a dry skin surface, with those of vernix caseosa, excreted to a wet fetal skin surface (18).

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REFERENCES

1. Nicolaides, N., and Ruth, E.C. (1982) Current Eye Res. 2, 93-98.
2. Nicolaides, N., Kaitaranta, J.K., Rawdah, T.N., Macy, J.I., Boswell, F.M., and Smith, R.E. (1981) Invest. Ophthalmol. Vis. Sci. 20, 522-536.
3. Kaitaranta, J.K., and Linko, R.R. (1979) J. Sci. Food Agric. 30, 921.
4. Siakotos, A.N., and Rouser, G. (1965) J. Am. Oil Chem. Soc. 42, 913-919.
5. McCarthy, R.D., and Duthie, A.H. (1962) J. Lipid Res. 3, 117-119.
6. Metcalfe, L.D., and Schmitz, A.A. (1961) Anal. Chem. 33, 363-364.
7. Farquhar, J.W. (1962) J. Lipid Res. 3, 21-30.
8. Vandenheuvel, F.A. (1956) Anal. Chem. 28, 362-365.
9. Stein, R.A., and Nicolaides, N. (1962) J. Lipid Res. 3, 476-478.
10. Demole, E., and Wuest, H. (1961) Chimia 15, 286-287.
11. Nicolaides, N. (1967) Lipids 2, 266-275.
12. Ackman, R.G. (1972) Progr. Chem. Fats Other Lipids 12, 233-235.
13. Apon, J.M.B., and Nicolaides, N. (1975) J. Chromatogr. Sci. 13, 467-473.
14. Nicolaides, N. (1974) Science 186, 19-26.
15. Kolattukudy, P.E., ed. (1976) Chemistry and Biochemistry of Natural Waxes, Chapters 2, 4 and 5, Elsevier Pub. Co., New York, NY.
16. Jacob, J. (1976) "Bird Waxes" Chemistry and Biochemistry of Natural Waxes, Chapters 4 and 5, Elsevier Pub. Co., New York, NY.
17. Brett, D., Howling, D., Morris, L.J., and James, A.T. (1971) Arch. Biochem. Biophys. 143, 535-547.
18. Nicolaides, N., Fu, H.C., Ansari, M.N.A., and Rice, G.R. (1972) Lipids 7, 506-517.

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Singlet Oxygen in Copper-Catalyzed Lipid Peroxidation in Erythrocyte Membranes

AI-HAO DING and **PHILLIP C. CHAN,*** *The Department of Biochemistry, State University of New York Downstate Medical Center, Brooklyn, NY 11203*

ABSTRACT

Lipid hydroperoxide was generated in human erythrocyte membranes by irradiation with near ultraviolet (UV) light in the presence of a photosensitizer, hematoporphyrin, but no production of 2-thiobarbituric acid-reactive materials (malonaldehyde and its precursors) was detected. Incubation of the irradiated membranes with $CuSO_4$ led to increased levels of hydroperoxide and formation of malonaldehyde. Hydroperoxides were essential for initiating the Cu(II)-catalyzed peroxidation as no significant activity was observed with nonirradiated membranes and Cu(II) unless an organic peroxide, either t-butyl hydroperoxide or cumene hydroperoxide, was added. Catalytic activity was also found with Fe(II), but not with other metal ions tested. The peroxidation catalyzed with Cu(II) was partially inhibited by several singlet oxygen quenchers but was not affected by superoxide dismutase, catalase or $OH^{\cdot}$ radical scavengers. The possible involvement of singlet oxygen in the Cu(II)-catalyzed peroxidation reaction was further supported by a 3-fold enhancement of malonaldehyde production in D_2O. *Lipids*, 19:278-284, 1984.

INTRODUCTION

The complexity of the mechanisms of lipid peroxidation is evident from numerous recent reports implicating various reactive oxygen species in this process. Some hypotheses are amply substantiated, whereas others are still not well understood. These oxygen species, including the $^1\Delta_g$ state of the singlet molecular oxygen ($^1O_2$) (1,2), $OH^{\cdot}$ (3-5), $O_2^{\cdot-}$ (6-8), $HO_2^{\cdot}$ (9), H_2O_2 (10,11) and metal-oxygen complexes (11-14), may be involved in lipid peroxidation directly or indirectly in various combinations. Metal ion-catalyzed peroxidation is mostly considered to be mediated by $OH^{\cdot}$ via the Fenton reaction (3-5,10,13-15). The participation of $^1O_2$ in some of the lipid peroxidation systems, however, is still controversial.

In this study we used a simple system to illustrate a 2-stage peroxidation mechanism in erythrocyte membranes. Lipid hydroperoxides were produced by photooxidation and, subsequently, Cu(II) served as a catalyst for the second stage of the peroxidation leading to malonaldehyde as one of the products. Attempts were made to examine the possible involvement of $^1O_2$ in the metal-catalyzed lipid peroxidation.

EXPERIMENTAL PROCEDURES

Preparation of Erythrocyte Membranes

Human blood was collected from healthy donors. Erythrocyte membranes were prepared with some modification (16) according to the procedure of Dodge et al. (17). The membrane suspensions, with 1 mg protein per ml, were stored at −70 C in a series of tubes.

Photooxidation

A standard reaction mixture, which contained erythrocyte membranes (1 mg of protein/ml), 5 μM hematoporphyrin, 7 mM sodium phosphate, pH 7.5, was incubated in a 37 C water bath with constant stirring and irradiated directly with two 15-watt Sylvania F15T8-BL blacklight fluorescent bulbs with a maximum emission of 375 nm (18). The light intensity was adjusted by the distance of the light from the reaction mixture and was determined with a Black-Ray long-wave ultraviolet (UV) meter (Ultraviolet Products Inc.). The light intensity used was 10 W/m^2 unless otherwise indicated.

Hydroperoxide Determination

Hydroperoxide content in erythrocyte membranes was determined iodometrically according to the procedure of Buege and Aust (19) and expressed as nmol hydroperoxide/mg membrane protein. A molar extinction coefficient of 1.73×10^4 at 353 nm (19) was used.

Cu(II)-Catalyzed Peroxidation and Malonaldehyde Determination

A standard reaction mixture containing an irradiated membrane sample with 0.5 mg protein, 5 mM sodium phosphate and 50 μM $CuSO_4$ in a total volume of 1.0 ml, pH 7.5, was incubated at 37 C. The reaction was stopped by the addition of 10 μl of 0.5 M ethylenediamine tetraacetic acid (EDTA) and 50 μl of 6.1 M

*To whom correspondence should be addressed.

trichloroacetic acid. The mixture was centrifuged at 2,000 x *g* in an International Refrigerated Centrifuge for 10 min. An aliquot of the supernatant (0.8 ml) was taken for thiobabituric acid assay according to the procedure of Bidlack and Tappel (20). The spectrum of the reaction products between 400 and 650 nm was identical to that of a standard malonaldehyde sample. Therefore, the thiobarbituric acid-reactive materials in the products were assumed to be malonaldehyde and its precursors. The results were expressed as equivalent amounts of malonaldehyde per mg membrane protein. The molar extinction of 1.56×10^5 at 532 nm (21) was used in the assay.

Materials

Hematoporphyrin dihydrochloride was obtained from Sigma Chemical Co., St. Louis, MO; 1,4-diazabicyclo [2,2,2]octane (DABCO) from Matheson, Coleman and Bell (Norwood, OH); 2,5-dimethylfuran (DMF) from Aldrich Chemical Co. (Milwaukee, WI); 2,5-diphenylfuran (DPF) from Eastman Kodak Co., Rochester, NY; 99.8% D_2O from Bio-Rad Laboratories (Richmond, CA); beef liver catalase from Boehringer Mannheim Biochemicals (Mannheim, W. Germany). All other chemicals were of reagent grade. Superoxide dismutase (SOD) was purified from bovine erythrocytes according to the procedure of McCord and Fridovich (22).

RESULTS

Production of Hydroperoxides during Photooxidation

That singlet oxygen can directly react with a double bond in an unsaturated fatty acid by a concerted addition to yield an allylic hydroperoxide has been well established (23-25). Figure 1 shows that when erythrocyte membranes were exposed to near UV light, in the presence of 5 μM hematoporphyrin, the accumulation of hydroperoxides increased throughout the course of irradiation. But no simultaneous production of malonaldehyde occurred, which suggests that oxidation of membrane lipid by singlet oxygen alone can lead to lipid hydroperoxides as relatively stable end products.

Cu(II)-Catalyzed Lipid Peroxidation

As shown in Figure 2, when irradiated membranes were incubated with 50 μM $CuSO_4$, malonaldehyde was formed continuously up to 120 min. No significant malonaldehyde production was found in an irradiated membrane sample incubated without Cu(II), a nonirradiated sample incubated with Cu(II), or a membrane sample irradiated in the absence of hematoporphyrin and then incubated with Cu(II). This suggests the requirement of some photooxidation products as precursors for malonaldehyde production.

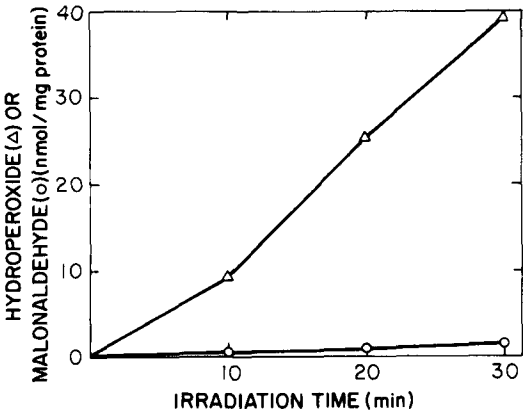

FIG. 1. Production of hydroperoxides by photooxidation. Erythrocyte membranes were irradiated for varying lengths of time and assayed for hydroperoxide (Δ) and malonaldehyde (○) contents, as described under Experimental Procedures.

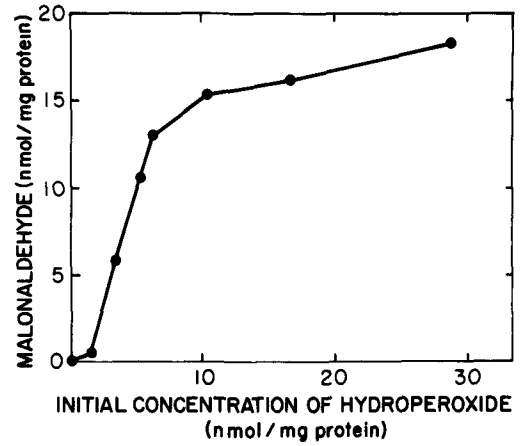

FIG. 2. Cu(II)-catalyzed peroxidation in irradiated membranes. Erythrocyte membranes were irradiated for 10 min, then incubated with (•) or without (○) 50 μM $CuSO_4$, as described under Experimental Procedures. Malonaldehyde produced during incubation with Cu(II) and either membranes irradiated in the absence of hematoporphyrin or nonirradiated membranes with hematoporphyrin is represented by (x).

Attempts were made to correlate the initial concentration of hydroperoxides formed during photooxidation with malonaldehyde production in subsequent incubation with Cu(II). After membrane samples were irradiated for varying lengths of time, their hydroperoxide contents were determined, and they were then

incubated with 50 μM $CuSO_4$ for 30 min. Figure 3 shows that the production of malonaldehyde increased proportionally to the initial hydroperoxide concentration up to 6 nmol/mg membrane protein, then leveled off gradually as it approached a saturation point. When the concentration of hydroperoxide was below 2 nmol/mg protein, no significant malonaldehyde production was detected. This may be because of the presence of endogenous antioxidants as observed in an early study on membrane lipid peroxidation in the presence of Cu(II) and H_2O_2, in which lipid peroxidation was also not detected at low concentrations of H_2O_2 (11).

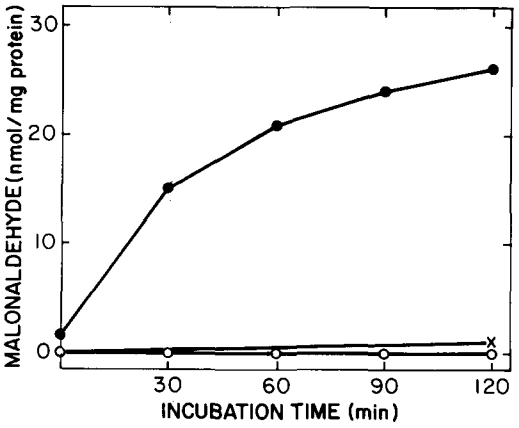

FIG. 3. Cu(II)-catalyzed malonaldehyde production as a function of initial hydroperoxide concentration. Erythrocyte membranes were irradiated at 8 W/m^2 for varying lengths of time. Hydroperoxide content was determined and duplicate irradiated samples were incubated with 50 μM $CuSO_4$ for 30 min. Malonaldehyde formed during the incubation of irradiated membranes with $CuSO_4$ was determined.

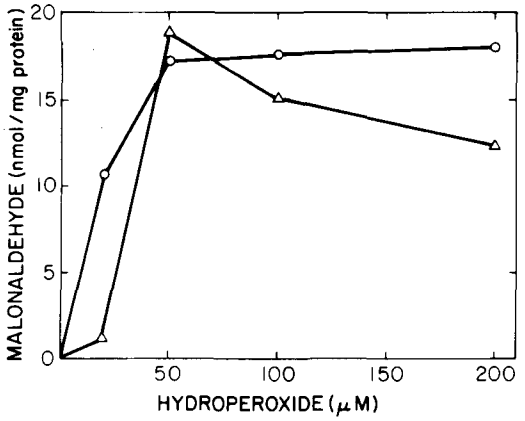

FIG. 4. Cu(II)-catalyzed membrane lipid peroxidation in the presence of either t-butyl hydroperoxide or cumene hydroperoxide. Erythrocyte membranes were preincubated with either t-butyl hydroperoxide (○) or cumene hydroperoxide (△) for 1 hr at 37 C, then incubated with 50 μM $CuSO_4$ for another 2 hr. Malonaldehyde production was determined as described under Experimental Procedures.

Requirement of Hydroperoxides for Malonaldehyde Production

Figure 4 provides additional support for the requirement of hydroperoxides in Cu(II)-catalyzed peroxidation. Incubation of non-irradiated membranes with either cumene hydroperoxide or t-butyl hydroperoxide in the presence of 50 μM $CuSO_4$ also led to production of malonaldehyde. Furthermore, the results indicate that hematoporphyrin, which was present in most of the other experiments, was not necessary for the hydroperoxide-dependent lipid peroxidation catalyzed by Cu(II).

Another experiment was carried out to further illustrate the requirement of hydroperoxides for malonaldehyde production. When irradiated membranes were treated with KI, 60% of the hydroperoxides were reduced. After removal of excess reagents by washing, the membranes were incubated with 50 μM $CuSO_4$. Production of malonaldehyde in the KI-treated sample was only ca. 20% of that in the control (data not shown).

Time Course of Cu(II)-Catalyzed Peroxidation

During the incubation of irradiated membranes with Cu(II), a continuous change in the concentrations of hydroperoxides and malonaldehyde was found, as shown in Figure 5. The

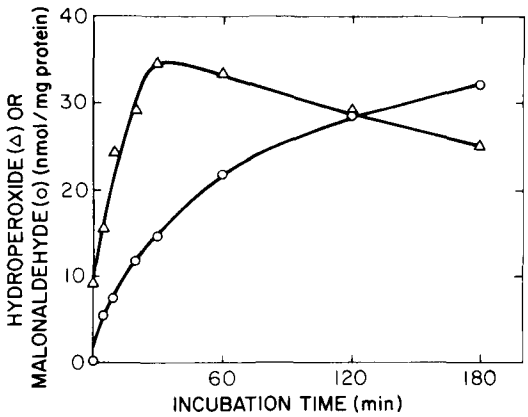

FIG. 5. Time course of Cu(II)-catalyzed lipid peroxidation. Erythrocyte membranes were irradiated for 10 min, then incubated with 50 μM $CuSO_4$ as described under Experimental Procedures. Aliquots were taken at different intervals, as indicated, and assayed for their hydroperoxide and malonaldehyde contents.

hydroperoxide content in the membrane increased ca. 3-fold in 30 min and declined gradually thereafter. Meanwhile, the production of malonaldehyde, starting from zero time, increased steadily throughout the incubation time of 3 hr.

Catalytic Activity of Cu(II) and Fe(II)

An earlier study (11) showed that Cu(II) was uniquely capable of catalyzing membrane lipid peroxidation in the presence of H_2O_2. Several other transition metal ions have also been implicated in lipid peroxidation in some other model systems (4,10,11,14).

Table 1 compares the catalytic activities of various transition metal ions on the peroxidation of irradiated erythrocyte membranes. Production of malonaldehyde was observed in catalysis with either Cu(II) or Fe(II), while the other metal ions tested were without significant activities. The addition of ADP did not potentiate the activity of either Fe(II) or Fe(III).

The metal ion receiving the most attention in lipid peroxidation is the ferrous ion, which is thought to play an important role in the Haber-Weiss reaction and the Fenton reaction. However, in our system, at the concentration of 0.05 mM, Cu(II) was twice as effective as Fe(II) in catalyzing peroxidation in irradiated membranes (Table 1).

The effects of Cu(II) and Fe(II) were compared at various concentrations in Figure 6. Cu(II), serving as a catalyst in the peroxidation, reached a maximum at 0.1 mM and remained at a plateau thereafter. On the other hand, the catalytic activity of Fe(II) increased steadily throughout the concentration range tested. The results appear to indicate that the mechanisms of catalysis by these 2 metal ions are quite different. Moreover, at low concentrations, close to physiological conditions (below 0.1 mM), the effect of Cu(II) is more pronounced than Fe(II).

TABLE 1

Effects of Metal Ions[a]

Metal ion[b]	Malonaldehyde production
(50 μM)	(nmol/mg protein)
$CuSO_4$	15.6
$FeSO_4$	8.6
$FeSO_4$ (plus 0.2 mM ADP)	8.5
$FeCl_3$	0.4
$FeCl_3$ (plus 0.2 mM ADP)	0.2
$SnCl_2$	0.1

[a]Erythrocyte membranes were irradiated for 10 min and then incubated for 30 min with various metal ions, as indicated. The procedures for irradiation, incubation and malonaldehyde assay were as described under Experimental Procedures.

[b]Metal ions tested without detectable effects included $CdCl_2$, $NiSO_4$, $CoCl_2$, $Cr(NO_3)_3$, $MnSO_4$, $ZnSO_4$ and $HgCl_2$.

Effects of Various Scavengers

The hydroxyl radical, superoxide radical and hydrogen peroxide have often been implicated in metal-catalyzed lipid peroxidation (6,8,11, 26-28). Table 2 shows that malonaldehyde production in Cu(II)-catalyzed peroxidation in the irradiated membranes was not affected by addition of scavengers of $OH^{\bullet}$ radicals such as formate, benzoate, mannitol or methanol. In addition, neither superoxide dismutase nor catalase inhibited the reaction. Thus, $OH^{\bullet}$, $O_2^{\bullet-}$ and H_2O_2 may be excluded as major mediators in the peroxidation. In contrast, a nonspecific free radical scavenger, either 2,6-ditert-butyl-p-cresol (BHT) or 2,(3)-tert-butyl-4-hydroxyanisole (BHA), was able to abolish malonaldehyde production, which implies the involvement of other free radicals, possibly peroxy and alkoxy radicals, in this process.

When $^1O_2$ scavengers, DABCO, DMF, DPF and NaN_3, were tested, they all showed partial inhibitory effects (Table 2), which suggested that $^1O_2$ may also be involved. However, as has been pointed out, these scavengers and quenchers for $^1O_2$ are not specific (29,30). To further examine its involvement by other means is necessary.

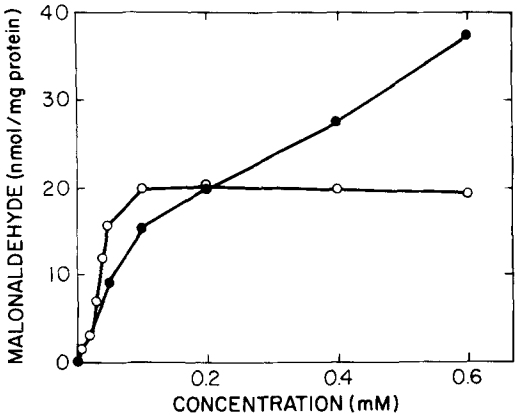

FIG. 6. Membrane lipid peroxidation as a function of Cu(II) or Fe(II) concentration. Erythrocyte membranes were irradiated for 10 min, and then incubated with varying concentrations of either $CuSO_4$ (○) or $FeSO_4$ (●) for 30 min. Malonaldehyde production was determined as described under Experimental Procedures.

TABLE 2

Effects of Scavengers on Malonaldehyde Production[a]

Addition	Concentration	Percentage of control
Sodium benzoate	20 mM	97
Sodium formate	20 mM	97
D-Mannitol	20 mM	102
Methanol	24 mM	102
SOD	20 μg/ml	100
Catalase	20 μg/ml	98
DMF	20 mM	42
DPF	2 mM	60
DPF	20 mM	46
DABCO	40 mM	76
NaN_3	10 mM	76
NaN_3	20 mM	67
NaN_3	30 mM	41
BHA	10 μM	3
BHT	10 μM	2

[a]Erythrocyte membranes were irradiated for 10 min. Various scavengers were added as indicated, and then the mixtures were incubated in the presence of 50 μM of $CuSO_4$ for 30 min. DMF, BHA and BHT were dissolved in methanol, and DPF in acetone. The equivalent amount of each solvent was added to the respective control. Details of the experiments were as described under Experimental Procedures except that the effect of DABCO was tested in 20 mM Tris buffer, pH 8.5.

Effect of D_2O on Photooxidation and on Cu(II)-Catalyzed Peroxidation

As the mean lifetime of $^1O_2$ in D_2O is more than 10 times longer than in H_2O (31), an enhancement of reactivity involving $^1O_2$ would be expected when D_2O is substituted for H_2O in the medium.

Because $^1O_2$ is known to be a mediator in hematoporphyrin-catalyzed photooxidation, we found, as expected, that irradiation of erythrocyte membrane in D_2O resulted in 3 times greater production of lipid hydroperoxides compared with that in H_2O (data not shown). An effect of D_2O was also found in the second stage of peroxidation. As shown in Figure 7, when a membrane sample was irradiated in H_2O and subsequently incubated with $CuSO_4$ in 95% D_2O, a 3-fold activation in malonaldehyde production occurred.

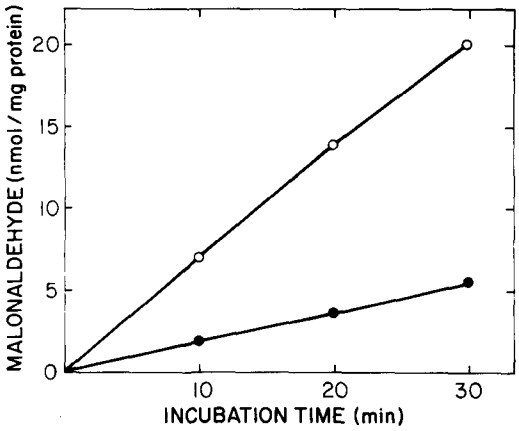

FIG. 7. Effect of D_2O in Cu(II)-catalyzed lipid peroxidation in irradiated membranes. Erythrocyte membranes were irradiated at 5 W/m² for 10 min in an H_2O medium. One-half of the sample was washed with D_2O buffer and finally incubated with 50 μM $CuSO_4$ in 95% D_2O (○). The other half was treated similarly in an H_2O medium (●).

DISCUSSION

Several recent studies have implicated involvement of singlet oxygen in membrane lipid peroxidation by $O_2^{\cdot-}$-generating systems such xanthine-xanthine oxidase (32-34) and microsomal NADPH cytochrome P_{450} reductase (33, 35-37). That $^1O_2$ could be produced from other oxygen species via interaction between $O_2^{\cdot-}$ and H_2O_2 (6,28,37), dismutation of $O_2^{\cdot-}$ (33) and breakdown of hydroperoxide through peroxy radicals (28,38) has been suggested. These interconversions among various reactive oxygen species, however, are still not well understood and remain controversial (39,40). The scavengers and quenchers for $^1O_2$ often used in these studies are not specific (29,30,41). Consequently, the evidence for the involvement of $^1O_2$ in a complex, enzyme-catalyzed lipid peroxidation has often been questioned (40, 42).

In the present study on the 2 stages of lipid peroxidation in erythrocyte membranes, a well-established $^1O_2$-generating system, hematoporphyrin-catalyzed photooxidation (2,18), was used in the first stage. Lipid hydroperoxides were generated by the irradiation of membranes in the presence of 5 μM hematoporphyrin, but no significant malonaldehyde production was found from the photooxidation (Fig. 1), suggesting that $^1O_2$ alone is not sufficient to cause chain oxidation leading to free-radical reaction products.

In the second stage, lipid hydroperoxides served as initiators for Cu(II)-catalyzed peroxidation. Further increases in lipid hydroperoxides and formation of malonaldehyde were found (Fig. 5). The requirement of hydroperoxide as an initiator was supported by the following results: (a) at low concentrations, the production of malonaldehyde was correlated with the initial concentration of lipid peroxides formed during photooxidation (Fig. 3); (b) when an irradiated membrane sample was

treated with KI to partially reduce the lipid peroxides, the subsequent production of malonaldehyde when incubated with Cu(II) was also decreased; and (c) when a nonirradiated membrane sample was incubated with Cu(II) in the presence of either t-butyl hydroperoxide or cumene hydroperoxide, lipid peroxidation was also detected by the production of malonaldehyde (Fig. 4).

The time course study in Figure 5 supports the hypothesis that lipid hydroperoxides are intermediates in the production of malonaldehyde (5). Within 30 min, the concentration of lipid hydroperoxides increased to a maximum and then declined gradually thereafter, whereas the amount of malonaldehyde accumulated continuously. The 3-fold increase of lipid hydroperoxide indicates that Cu(II)-catalyzed lipid peroxidation is not a simple conversion of hydroperoxide to malonaldehyde. It also includes a propagation of a chain reaction in the production of more hydroperoxides as intermediates.

Involvement of $^1O_2$ in Cu(II)-catalyzed peroxidation was supported by the effects of D_2O and $^1O_2$ scavengers. When a sample of irradiated membranes in a H_2O medium was washed and subsequently resuspended and incubated with Cu(II) in a D_2O medium, up to 3-fold activation of malonaldehyde production was observed (Fig. 7). The $^1O_2$ scavengers, which exerted significant inhibitory effects in Cu(II)-catalyzed peroxidation, included DABCO, DMF, DPF and azide (Table 2). However, none of the $^1O_2$ scavengers, even at relatively high concentrations, inhibited malonaldehyde production completely (Table 2). It implies that a fraction of the malonaldehyde produced was generated by a free-radical peroxidation mechanism that did not require $^1O_2$ as a mediator.

In order to study the possible involvement of $OH^\bullet$ and other potential derivatives from H_2O_2 and/or $O_2^{\bullet-}$, the effects of catalase, SOD and several well known scavengers for $OH^\bullet$ were examined. Neither $O_2^{\bullet-}$ nor H_2O_2 appears to play an important role in the Cu(II)-catalyzed peroxidation, as no significant difference was observed in the presence of either SOD or catalase (Table 2). Competitive studies with $OH^\bullet$ scavengers, including mannitol, formate, benzoate and methanol, also did not show any detectable effect (Table 2). In other systems in which $O_2^{\bullet-}$ and H_2O_2 are thought to be involved to generate $OH^\bullet$ in the Fenton reaction, inhibition of lipid peroxidation by catalase, SOD or $OH^\bullet$ scavengers was observed (15,32, 34,43). Thus, in this system, the $OH^\bullet$ radical is not likely to be a major mediator. However, the possibility of generation of any of these species right at the site of reaction is not excluded, thus eluding the effect of a scavenger or a protective enzyme in the aqueous medium.

Russell (44) postulated a mechanism for the conversion of peroxy radicals to alkoxy radicals accompanied by the production of molecular oxygen. Hawco et al. (26) suggested that the oxygen product might be in the singlet state based on the chemiluminescence they observed during the ceric ion-catalyzed decomposition of linoleic acid hydroperoxide. Nakano et al. (38) cited the same mechanism in their spectroscopic studies on the decomposition of organic peroxy radicals. Possible generation of $^1O_2$ from lipid hydroperoxide was also indicated in the study of Lai et al. (45) using DPF as a trap. Perhaps a similar mechanism may also be involved in the hydroperoxide-dependent lipid peroxidation catalyzed by Cu(II) in the erythrocyte membrane. The metal ion could react with hydroperoxides to form peroxy radicals (46) and thus lead to further generation of $^1O_2$ which, in turn, reacts with unsaturated fatty acids to create additional hydroperoxides. Cu(II) may also catalyze the decomposition of peroxy radicals to produce malonaldehyde.

This study clearly demonstrates a two-step mechanism of lipid peroxidation in erythrocyte membranes: hematoporphyrin-sensitized photooxidation and Cu(II)-catalyzed, hydroperoxide-dependent free-radical chain reaction leading to malonaldehyde production. The results suggest that $^1O_2$ plays a partial role in the hydroperoxide-dependent lipid peroxidation catalyzed by Cu(II). They also indicate that $^1O_2$ by itself is not capable of propagating the chain peroxidation reaction to generate malonaldehyde.

ACKNOWLEDGMENT

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REFERENCES

1. Frankel, E.N., Neff, W.E., and Bessler, T.R. (1979) Lipids 14, 961-967.
2. Nickon, A., and Bagli, J.F. (1960) J. Am. Chem. Soc. 83, 1498-1508.
3. Fong, K., McCay, P.B., Pryer, J.L., Keele, B.B., and Misra, H. (1973) J. Biol. Chem. 248, 7792-7797.
4. Tien, M., Svingen, B.A., and Aust, S.D. (1982) Arch. Biochem. Biophys. 216, 142-151.
5. Bus, J.S., and Gibson, J.E. (1979) Rev. Biochem. Toxicol. 1, 125-149.
6. Kellogg, E.W., III, and Fridovich, I. (1977) J. Biol. Chem. 252, 6721-6728.
7. Sutherland, M.W., and Gebicki, J.M. (1982) Arch. Biochem. Biophys. 214, 1-11.
8. Kumar, K.S., Rowse, C., and Hochstein, P. (1978) Biochem. Biophys. Res. Commun. 83, 587-592.

9. Gebicki, J.M., and Bielski, B.H.J. (1981) J. Am. Chem. Soc. 103, 7020-7021.
10. Lai, C.S., and Piette, L.H. (1978) Arch. Biochem. Biophys. 190, 27-38.
11. Chan, P.C., Peller, O.G., and Kesner, L. (1982) Lipids 17, 331-337.
12. Fee, J.A. (1980) in Metal Ion Activation of Dioxygen (Spiro, T.G., ed.) pp. 209-237, John Wiley and Sons, Inc., New York.
13. Fridovich, S.E., and Porter, N.A. (1981) J. Biol. Chem. 256, 260-265.
14. Mead, J.F. (1976) in Free Radicals in Biology (Pryor, W.A., ed.) Vol. I, pp. 51-68, Academic Press, New York.
15. Tien, M., Svingen, B.A., and Aust, S.D. (1981) Fed. Proc. 40, 179-182.
16. Kesner, L., Kindya, R.J., and Chan, P.C. (1979) J. Biol. Chem. 254, 2705-2709.
17. Dodge, J.T., Mitchell, C., and Hanahan, D.J. (1963) Arch. Biochem. Biophys. 100, 119-130.
18. Bodaness, R.S., and Chan, P.C. (1977) J. Biol. Chem. 252, 8554-8560.
19. Buege, J.A., and Aust, S.D. (1978) Methods Enzymol. 52, 302-310.
20. Bidlack, W.R., and Tappel, A.L. (1973) Lipids 8, 177-182.
21. Wills, E.D. (1969) Biochem. J. 113, 315-324.
22. McCord, J.M., and Fridovich, I. (1969) J. Biol. Chem. 244, 6049-6055.
23. Frankel, E.N. (1980) Prog. Lipid Res. 19, 1-22.
24. Thomas, M.J., and Pryor, W.A. (1980) Lipids 15, 544-548.
25. Terao, J., Hirota, Y., Kawakatsu, M., and Matsushita, S. (1981) Lipids 16, 427-432.
26. Hawco, F.J., O'Brien, C.R., and O'Brien, P.J. (1977) Biochem. Biophys. Res. Commun. 76, 354-361.
27. Gutteridge, J.M.C. (1977) Biochem. Biophys. Res. Commun. 77, 379-386.
28. Kobayashi, Y., and Usui, T. (1982) Biochem. Biophys. Res. Commun. 105, 539-545.
29. Borg, D.C., Schaich, K.M., Elmore, J.J., Jr., and Bell, J.A. (1978) Photochem. Photobiol. 28, 887-907.
30. Packer, J.E., Mahood, J.S., Mora-Arellano, V.O., Slater, T.F., Willson, R.L., and Wolfenden, B.S. (1981) Biochem. Biophys. Res. Commun. 98, 901-906.
31. Merkel, P.B., and Kearns, D.R. (1972) J. Am. Chem. Soc. 94, 7244-7253.
32. Pederson, T.C., and Aust, S.D. (1975) Biochim. Biophys. Acta 385, 232-241.
33. Svingen, B.A., O'Neal, F.O., and Aust, S.D. (1978) Photochem. Photobiol. 28, 803-809.
34. Lynch, R.E., and Fridovich, I. (1978) J. Biol. Chem. 253, 1838-1845.
35. King, M.M., Lai, E.K., and McCay, P.B. (1975) J. Biol. Chem. 250, 6496-6502.
36. Nakano, M., Noguchi, T., Sugioka, K., Fukuyama, H., and Sato, M. (1975) J. Biol. Chem. 250, 2404-2406.
37. Dixit, R., Mukhtar, H., and Bickers, D.R. (1982) Biochem. Biophys. Res. Commun. 105, 546-552.
38. Nakano, M., Takayama, K., Shimizu, Y., Tsuji, Y., Inaba, H., and Migita, T. (1976) J. Am. Chem. Soc. 98, 1974-1975.
39. Foote, C.S., Shook, F.C., and Abakerli, R.A. (1980) J. Am. Chem. Soc. 102, 2503-2504.
40. Smith, L.L., and Teng, J.I. (1974) J. Am. Chem. Soc. 96, 2640-2641.
41. Takayama, K., Noguchi, T., Nakano, M., and Migita, T. (1977) Biochem. Biophys. Res. Commun. 75, 1052-1058.
42. Thomas, M.J., Mehl, K.S., and Pryor, W.A. (1982) J. Biol. Chem. 257, 8343-8347.
43. Miura, T., and Ogiso, T. (1982) Chem. Pharm. Bull. 30, 3662-3668.
44. Russell, G.A. (1957) J. Am. Chem. Soc. 79, 3871-3877.
45. Lai, E.K., Fong, K., and McCay, P.B. (1978) Biochim. Biophys. Acta 528, 497-506.
46. Black, J.F. (1978) J. Am. Chem. Soc. 100, 527-535.

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Fatty Acid Desaturase Specificity in Tetrahymena

ROBERT L. CONNER,[a]* BARBARA BURTNESS,[a] and KAREN A. FERGUSON,[b]
[a]*Department of Biology, Bryn Mawr College, Bryn Mawr, PA 19010; and* [b]*Department of Biochemistry, SUNY at Buffalo, Buffalo, NY 14214*

ABSTRACT

The ciliate, *Tetrahymena,* was provided a supplement of the fatty acid [1-^{14}C] 18:2Δ6,9. After a period of growth the cells were claimed, the lipids extracted, the polar lipids recovered and the mild alkali-labile fatty acid methyl esters generated. The fatty acids were resolved by high pressure liquid chromatography (HPLC), the 18:3Δ6,9,12 (γ-linolenic acid) was recovered and its identity verified by high pressure liquid chromatography (HPLC), gas liquid chromatography (GLC), hydrogenation and oxidation. Fifty-three percent of the cell-associated label was found in γ-linolenic acid; thus, a Δ12 fatty acid desaturase converts the 6,9 octadecadienoic acid to the 6,9,12 derivative. No carboxyl or methyl terminus restriction appears on Δ9 monoenoic or dienoic fatty acid desaturation in this cell as is found in higher plants and animals.
Lipids, 19:285-288, 1984.

Unsaturated fatty acids are synthesized in *Tetrahymena* through a series of steps that give rise to a mixture of 9-monoenoic fatty acids, both 9,12- and 6,9-dienoic acids, and finally the 6,9,12-trienoic acids (Fig. 1) (1-7). These reactions are carried out by fatty acid desaturases that probably include 3 separate enzymes, with specificity for placement of the double bond at C-6, C-9 and C-12. As is the general case for eukaryotic cells, the first double bond inserted in *Tetrahymena* fatty acids is that at C 9,10. The specificity of the 9 desaturase has been studied extensively in vivo and limited in vitro investigations with palmitic and stearic acids have been performed (8,9). Normal fatty acids of chain length C_{15-19} can serve as substrates for this enzyme, with chain lengths of C_{17-19} preferred. Branched chain (iso) acids are less effective substrates, but C_{16-19} monoenoic acids are formed (3,6). Anteiso branched chain acids are not substrates for the 9 desaturase (6).

The array of polyunsaturated fatty acids in *Tetrahymena* indicates that the 6 desaturase can use a variety of normal fatty acids as substrates. Δ9 Monoenes are desaturated to 6,9 dienes (4,7), an 11 monoene is desaturated to a 6,11 diene (4,10) and 9,12-dienes of C_{16-19} chain length give rise to 6,9,12-trienoic acids (1,4,10). Branched chain 9 monounsaturated acids are not further desaturated in this organism, presumably because the bulk of the methyl group prevents proper binding to the enzyme

Our knowledge about substrate specificity of the Δ12 desaturation step is more limited. The introduction of a double bond into 9 monoenoic acids to give rise to the corresponding 9,12 dienes is the only previously known reaction in

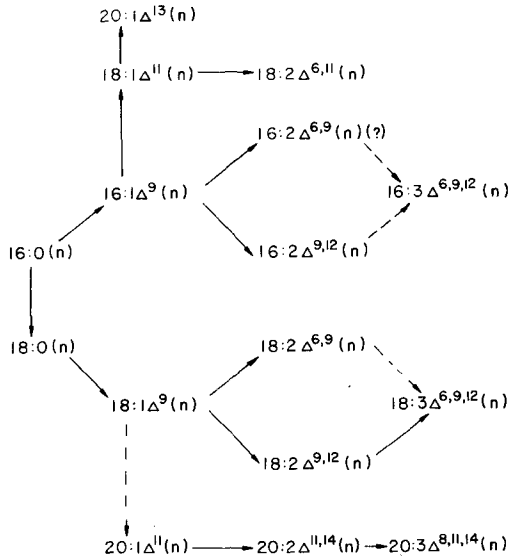

FIG. 1. Proposed scheme for the synthesis of unsaturated fatty acids in *Tetrahymena.* The solid arrows indicate reactions that have been demonstrated and the dashed arrows those sequences that are assumed; (n), normal. The information in this figure can be found in references 1, 2, 4 and 10.

Tetrahymena, taking place on normal fatty acids of chain lengths of C_{16-19} (1,2,10). No 12 monoenes have been detected, so that the Δ12 desaturase apparently is not active on saturated fatty acids as substrates. Branched-chain acids are not substrates for this enzyme.

The lack of availability of radiolabeled 6,9 dienoic fatty acids has deterred resolution of the question of the suitability of such compounds as substrates for the 12 desaturase in

*To whom correspondence should be addressed.

Tetrahymena. An unsaturated fatty acid auxotroph of *Tetrahymena* (RH179E1) has been isolated that can be grown with an extensive series of unsaturated fatty acids (11). This mutant is deficient in both 9- and 12-desaturase activity, although a functional 6 desaturase is present (12). The 9 monoene, oleic acid, therefore, is converted in high yield to *cis,cis* 18:2Δ6,9. This property of the mutant allowed the preparation of the labeled diene that could be used, in turn, as a supplement with the wild cells to determine whether this compound is effectively converted by the 12 desaturase to 18:3Δ6,9,12.

MATERIALS AND METHODS

Preparation of Radiolabeled 6,9 Octadecadienoic Acid

Two 500 ml cultures of *T. thermophila* (RH179E1) were grown in 2% pepticase (Shefield, Inc.; Nashville, TN), 0.1% yeast extract (Difco) and 45 μM Fe:EDTA (ethylenediamine tetraacetic acid) chelate (13). Cultures were supplemented with 5 μC; [1-^{14}C]oleic acid (Amersham, Inc.) (sp.act. 57.4 mCi/mmol) and incubated for 48 hr at 28.5 C. An additional 3-cultures without the radiosupplement were used as a source of carrier lipid. The ciliates were claimed by continuous-flow centrifugation (14), lyophilized and extracted thrice with warm chloroform/methanol (2:1, v/v) (4). The extracts were combined and passed through a sintered glass filter. The solvents were removed by rotary evaporation under reduced pressure. Nonlipid contaminants were eliminated by the use of a gel filtration column (15) and the purified lipids were resolved into neutral and polar fractions by Unisil column chromatography (4). Free fatty acids (FFA) were released from the polar lipids by heating under nitrogen for 30 min at 75 C in 10% KOH in 80% methanol/20% water. The fatty acids were extracted after acidification with HCl into diethyl ether, and concentrated. Phenacyl esters were prepared by the method of Engelhardt and Elgass (16). The derivatives were injected directly onto the high pressure liquid chromatography (HPLC) column. HPLC separations were carried out on a Beckman Model 332 Gradient Liquid Chromatograph equipped with a Hitachi Model 100-10 variable wavelength spectrophotometer, and a 5 μm Ultrasphere ODS reversed phase column. HPLC grade water and acetonitrile were from J. T. Baker. Elution of phenacyl esters was performed with 80% acetonitrile/water (v/v) for 45 min at a flow rate of 2 ml/min, followed by acetonitrile for an additional 25 min, as described by Borch (17). Esters were detected by absorption at 254 nm, and collected individually for further analysis. To verify peak identifications, phenacyl esters were concentrated from the HPLC solvent by evaporation under nitrogen and converted to methyl esters by treatment with 0.5 N HCl in methanol heated under nitrogen for 30 min at 75 C. Gas liquid chromatography (GLC) was performed on a 6-ft column packed with 15% HI-Eff-BP (Applied Science) in a Varian 2100 chromatograph. The retention times of methyl esters were compared with those previously obtained (4). Radiolabeled samples were dried with a stream of nitrogen, dispersed in 5 ml Liquiscint (National Diagnostics) and counted in a Packard 3255 Liquid Scintillation Spectrometer.

Hydrogenation

Purified samples of 18:2Δ6,9 and 18:3Δ6,9, 12 methyl esters were subjected to catalytic hydrogenation with platinum oxide.

Double Bond Positioning

The purified fatty acid methyl ester samples were subjected to periodate-permanganate oxidation, and the monoenoic fragments were analyzed directly by GLC (4). The dicarboxylic fragments were methylated with diazomethane and chromatographed (4). Identification of the fragments was made by cochromatography with authentic standards (4,10). When aliquots of purified 18:2Δ6,9 were oxidized, the presence of the monocarboxylic acid, nonanoate and the dicarboxylic acid fragment, adipate, verified our earlier report (4) and established that the purified preparation contained less than 5% contamination by the 9,12 isomer, as evidenced by the lack of azaleic acid after oxidation.

The purified radiolabeled 18:2Δ6,9 (1.9×10^6 dpm/13.4 μmol) was added step by step to 2, 500 ml cultures of *T. pyriformis* W. After a 40 hr incubation at 28.5 C, the cells were claimed and subjected to the procedures described above. The 18:3Δ6,9,12 was isolated by HPLC, its identity was verified by GLC and an aliquot was assessed for radioactivity. A second portion was hydrogenated and found to yield only stearic acid (18:0). Periodate-permanganate oxidation produced hexanoic and adipic acids, confirming the identity of the trienoic acid.

RESULTS AND DISCUSSION

Three octadecadienoic acid isomers have been identified in *Tetrahymena sp:* 18:2Δ6,9, 18:2Δ9,12 and 18:2Δ6,11 are found in a mass ratio of 1:8:3 in *T. pyriformis* W (4). The first 2 are formed from oleic acid, whereas the last

originates via the palmitoleic acid pathway (Fig. 1) (10). In wild cells 18:2Δ9,12 predominates, whereas in a mutant of *T. thermophila* (RH179E1) that is defective in Δ9 and Δ12 desaturation, the 6,9 isomer is most abundant when oleate is provided as a supplement (12).

The retention times relative to methyl stearate on GLC (12% DEGS) of the methyl esters of the octadecadienoic isomers are: 6,11 (1.35); 6,9 (1.40); 9,12 (1.47). Chromatography on silver nitrate impregnated Unisil developed with increasing concentrations of benzene in petroleum ether show an elution pattern of 9,12 < 6,9 < 6,11. This procedure resolves the 6,11 isomer from the other two. HPLC of the phenacyl esters differs in the order of elution. The 9,12 phenacyl ester emerges first, followed closely by the 6,11 derivative; the 6,9 isomer is cleanly separated from the other two under the conditions employed and emerges last. γ-Linolenoyl phenacyl ester and oleoyl phenacyl esters elute ahead of and behind octadecadienoyl esters, respectively (Table 1).

TABLE 1

HPLC Retention Times Relative to 18:3 (1.00) of the Phenacyl Fatty Acids Esters Derived from the Glycerophospholipids of *Tetrahymena*

Fatty acid	RRT
12:0	0.71
18.3	1.00
14:0	1.29
16:1	1.35
18:2Δ9,12	1.47
18:2Δ6,9	1.62
16:0	2.41
18:1	2.47

Phenacyl esters prepared from the polar lipids of the mutant, RH179E1, grown with [1-^{14}C]oleic acid were chromatographed on HPLC and the 18:2Δ6,9 peak collected. The radioactivity recovered was 1.9×10^6 dpm. This ester was hydrolyzed to FFA and added to cultures of *T. pyriformis* W. After a prolonged incubation, the polar lipids were isolated, and FFA were obtained by alkaline hydrolysis followed by conversion to phenacyl esters and analysis by HPLC (Table 1). The fractions corresponding to 18:2Δ6,9 and 18:3Δ6,9,12 were collected separately, and the radioactivities were ascertained. Recoveries of the total dpm applied to the column were 95% or better. Table 2 shows the proportions of radioactive compounds eluted from HPLC. Clearly, a significant portion of the 18:2Δ6,9 was converted to γ-linolenate. Contamination of the starting material (18:2Δ6,9) by 18:2Δ9,12 was less than 5% as determined by analysis of the oxidation fragments and by HPLC, thus, could not account for the conversion observed. The 12 desaturase can use an appropriate 6,9 dienoic acid as a substrate in addition to the 9 monoenes.

TABLE 2

Distribution of Radioactivity in the Fatty Acids of the Polar Lipids of *Tetrahymena* Incubated with 1-[^{14}C]18:2Δ6,9

Fatty acid[a]	% of Radioactivity
18:1	<0.1
18:2Δ6,9	43
18:3Δ6,9,12	53

[a]The fatty acids were recovered from the polar lipids, converted to the phenacyl esters, resolved by HPLC and the radioactivity determined.

We may conclude that in *Tetrahymena*, no absolute specificity exists in the sequence of insertion of double bonds into long-chain normal acids other than that 9 desaturation must occur first. The 9 monoenes are suitable substrates for both the 6 and the 12 desaturases, although 9,12 dienes are found in larger amounts in this ciliate than the 6,9 isomers (1,2,4). C_{16-19} dienoic acids have been noted in *Tetrahymena* and, whereas only the 18 carbon acids have been shown to undergo further desaturation, that the other chain length 6,9 dienes also could serve as minor substrates for the 12 desaturase is a reasonable assumption. No obligatory carboxyl or methyl terminus constraint on further desaturation occurs as found in higher plants and animals.

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REFERENCES

1. Erwin, J., and Bloch, K. (1963) J. Biol. Chem. 238, 1618-1624.
2. Lees, A.M., and Korn, E.D. (1966) Biochemistry 5, 1475-1481.
3. Conner, R.L., Koo, K., and Landrey, J.L. (1974) Lipids 9, 554-559.
4. Ferguson, K.A., Davies, F.M., Conner, R.L., and Mallory, F.B. (1975) J. Biol. Chem. 250, 6998-7005.
5. Conner, R.L., and Reilly, A.E. (1975) Biochim. Biophys. Acta 398, 209-216.
6. Ragona, D.G., Cassel, D.L., Harnick, V.M., and Conner, R.L. (1981) Biochim. Biophys. Acta 663, 134-142.

7. Cassel, D.L., Ragona, D.G., Carriero, L., Kempe, J.A., and Conner, R.L. (1981) Biochim. Biophys. Acta 663, 121-133.
8. Nozawa, Y., and Kasai, R. (1978) Biochim. Biophys. Acta 529, 54-66.
9. Shapiro, H., and Prescott, D.J. (1978) Comp. Biochem. Physiol. 61B, 513-520.
10. Koroly, M.J., and Conner, R.L. (1976) J. Biol. Chem. 251, 7588-5792.
11. Hill, R. (1980) Biochim. Biophys. Acta 595, 140-145.
12. Hill, R., Prescott, D.J., Spence, S.L., and Conner, R.L. (1983) Biochim. Biophys. Acta 754, 150-158.
13. Conner, R.L., and Cline, S.G. (1964) J. Protozool. 11, 486-491.
14. Conner, R.L., Cline, S.G., Koroly, M.J., and Hamilton, B. (1966) J. Protozool. 13, 377-379.
15. Wuthier, R.E. (1966) J. Lipid Res. 7, 558-561.
16. Engelhardt, H., and Elgass, H. (1978) J. Chromatogr. 158, 249-259.
17. Borch, R.F. (1975) Anal. Chem. 47, 2437-2439.

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Phospholipid Synthesizing Enzymes of Dermatophytes III. Glycerol Kinase of Dermatophytes

C. KASINATHAN and G.K. KHULLER,* *Department of Biochemistry, Postgraduate Institute of Medical Education and Research, Chandigarh-160 012, India*

ABSTRACT

Glycerol kinase, the key enzyme for glycerol use in phospholipid synthesis, was identified in cytosolic fractions of 2 dermatophytes, *Microsporum gypseum* and *Epidermophyton floccosum*. Ammonium sulfate was observed to activate and stabilize this enzyme in both dermatophytes. Two pH optima, 8.0 and 10.5, were observed for both dermatophyte enzymes. Glycerol kinase from *M. gypseum* was purified up to 33-fold with a 225% recovery by ammonium sulfate precipitation and gel filtration. The molecular weight of the enzyme was ca. 4.5×10^5. It had 2 pH optima of 8.0 and 10.5. The enzyme had Km values of 0.35 mM and 2.3 mM for glycerol and ATP. Reactivity of the enzyme for various nucleotides was ATP > TTP > GTP > ITP = CTP = UTP. Kinetic studies showed the enzyme to catalyze the reaction by the ping-pong mechanism. Fructose 1,6-bisphosphate and glucose-6-phosphate inhibited the enzyme competitively, whereas glucose was not inhibitory.

INTRODUCTION

Dermatophytes are responsible for a skin infection called dermatomycosis, and phospholipid components of these organisms have been shown to elicit allergic reactions (1). Synthesis of membrane phospholipids in the host have been examined in detail and a key role has been assigned to glycerol kinase (2). This enzyme is responsible for glycerol-3-phosphate synthesis and has been characterized from rat liver (2), *Candida mycoderma* (3) and *Escherichia coli* (4), but not from filamentous fungi (5). Our laboratory is engaged in examining the biosynthesis of phospholipids in dermatophytes (6-10) and, in continuing our work on phospholipid synthesizing enzymes in dermatophytes, we have characterized glycerol kinase in 2 dermatophytes, i.e., *Microsporum gypseum* and *Epidermophyton floccosum*. Dermatophyte glycerol kinase was observed to be activated and stabilized by ammonium sulfate. This property had not been reported previously. In view of the identical kinetics exhibited by the crude enzyme from both the dermatophytes, we have purified this enzyme only from *M. gypseum* and the difference in its property compared with glycerol kinase of other sources has been discussed in this communication.

MATERIALS AND METHODS

Chemicals

[U-^{14}C]Glycerol (8 mCi/mmol) was obtained from New England Nuclear Corporation, Boston, MA. Dowex 1 x 8 Cl^- (100-200 mesh) ion exchange resin, disodium ATP and other nucleotides were obtained from Sigma Chemical Co., St. Louis, MO. Sephadex G-200 and high molecular weight kit were products of Pharmacia Fine Chemicals, Sweden.

Organisms and Growth Conditions

M. gypseum was obtained from the Public Health Laboratory Service, School of Hygiene and Tropical Medicine, London, U.K. The source and maintenance of *M. gypseum* and *Epidermophyton floccosum* were as given in our earlier study (7).

Subcellular Distribution

Cells in the log phase were harvested by filtration and washed with chilled normal saline to remove the adhering medium. Washed cells (15 g wet wt/100 ml buffer) were homogenized in a mortar in 10 mM Tris-HCl, pH 8.0, containing 250 mM sucrose. The homogenate was sonicated for 5 min (1 min at a time) by a Sonifier cell disruptor (Model W-195, manufactured by Branson Sonic Power Co.) at 55 watts. The temperature was maintained at 4 C. The homogenate was then centrifuged at 5,000 x *g* for 20 min to remove cell debris. Cell fractionation was carried out as described earlier (7). The 5,000 x *g* supernatant was spun at 15,000 x *g* for 30 min and the pellet was suspended in buffer by sonication, whereas the supernatant was centrifuged at 105,000 x *g* for 90 min. The pellet so obtained was suspended in the same buffer and sonicated for 15 seconds. The 15,000 x *g* and 105,000 x *g* pellets were characterized by assaying marker enzymes like cytochrome C-oxidase for the mitochondria and glucose 6-phosphatase for the microsomes, as described earlier (7).

*To whom correspondence should be addressed.

Protein Estimation

Protein was estimated by Lowry's method (11), using bovine serum albumin as a standard.

Ammonium Sulfate Fractionation

Because glycerol kinase activity was exclusively located in the 105,000 × *g* supernatant, ammonium sulfate was added to this fraction for 60% saturation. After stirring for 40 min, the pellet was collected by centrifugation at 25,000 × *g* for 30 min and dissolved in buffer A (10 mM Tris-HCl, pH 8.0, containing 250 mM and 400 mM ammonium sulfate).

Gel Filtration Chromatography

A Sephadex G-200 column (2.5 × 55 cm) was prepared and equilibrated with buffer A. The 60% ammonium sulfate precipitate was applied on the column and eluted with the same buffer. Fractions (5 ml each) were collected at a flow rate of 15 ml/hr. The active fractions were pooled and stored at −70 C until required.

Molecular Weight Determination

To determine the molecular weight, the enzyme was applied on a Sephadex G-200 column (2.5 × 55 cm) and eluted as mentioned above. Aldolase, catalase, ferritin and thyroglobulin were used as calibration proteins.

Enzyme Assay

Glycerol kinase activity was measured by the method of Jungries et al. (12) with slight modifications as described earlier (10). The enzyme was assayed by measuring the formation of radiolabeled glycerol 3-phosphate from [U-^{14}C]glycerol). The standard assay was done at 37 C for 60 min in a final volume of 1 ml, and the assay mixture contained 120 mM Tris-HCl (pH 8.0), 15 mM $MgCl_2$, 10 mM ATP, 2.5 mM [U-^{14}C]glycerol (0.25 mCi/mmol) and 0.3-0.5 mg purified enzyme protein. The reaction was stopped by adding 2 ml 100 mM glycerol in 0.2 N formic acid. An aliquot, 200 µl, of sample was applied to a column (0.5 × 5 cm) on Dowex-1-$HCOO^-$ × 8 (100-200 mesh ion exchange resin). The chloride form of resin was converted to its formate form by equilibrating it with 1 M sodium formate before applying the sample. The column was washed 3 times with 5 ml water, and radioactive glycerol 3-phosphate was eluted with 1 ml of 1 M ammonium formate and counted in Bray's fluid. Quenching correction was made by treating a known amount of radioactive glycerol with ammonium formate, as given above. A unit of glycerol kinase activity is defined as the amount in nmol of glycerol 3-phosphate formed per mg protein/hr. In kinetic studies, the concentrations of ATP and glycerol varied, as discussed later. To study nucleoside triphosphates affinity toward the enzyme, 10 mM ATP was replaced by an equal concentration of other nuceotides (GTP, CTP, UTP, ITP or TTP).

Product Identification

An aliquot of the assay mixture applied to a Whatman No. 1 paper and ascending chromatography was performed for 16-18 hr with n-butanol/acetic acid/water (5:2:3, v/v/v). The autoradiography of the chromatogram was done by exposing the chromatogram to Kodak X-ray film for 10-15 days. Detection of radioactive glycerol 3-phosphate in tests and its absence in the control confirmed the presence of glycerol kinase in the dermatophytes.

RESULTS AND DISCUSSION

Our earlier studies dealing with the biosynthesis of dermatophyte phospholipids revealed the presence of phosphatidic acid phosphatase (7) and choline kinase (8). Further, the presence of cytidine pathway for phosphatidylcholine (PC) synthesis and phosphatidylserine (PS)-decarboxylase and ethanolamine exchange reactions for phosphatidylethanalamine (PE) synthesis has also been demonstrated in our laboratory (6). This study presents the property of glycerol kinase in dermatophytes. This enzyme was mainly located in the cytosolic fraction (Table 1) and exhibited 2 optimum pH, i.e., 8.0 and 10.5, with crude preparation. Other

TABLE 1

Subcellular Distribution of Glycerol Kinase in *M. gypseum* and *E. floccosum*

Fractions	Specific activity (units/mg protein)	
	M. gypseum	*E. floccosum*
Mitochondrial	6.7 ± 1.2 (87.1)	7.85 ± 1.7 (59.55)
Microsomal	4.8 ± 1.5 (145.2)	4.54 ± 1.3 (25.1)
Cytosolic	23.82 ± 3.6 (2274.8)	14.81 ± 1.9 (2286.4)

Values are mean ± SD of 4 independent determinations.

One unit of the enzyme is defined as the nmol of glycerol 3-phosphate formed per mg protein per hr. Total activity of the enzyme is given in parentheses and 1 unit of total activity is defined as the nmol of glycerol 3-phosphate formed per hr.

enzyme kinetics, e.g., specificity to nucleotides, activation and stabilization of the enzymes by ammonium sulfate were also observed to be similar for this enzyme. Because the kinetic properties of both dermatophyte enzymes were same, the purification of glycerol kinase from *M. gypseum,* starting with its cytosolic fractions, was undertaken.

A cytosolic fraction with 60% ammonium sulfate gave a 10-fold purified enzyme in the precipitate (Table 2). Dialysis of this fraction for 18 hr resulted in a complete loss of enzyme activity, suggesting the requirement of ammonium sulfate for enzyme stability. Enzyme activity in the presence and absence of ammonium sulfate was examined at different intervals at 4-6 C (Fig. 1). The presence of ammonium sulfate stabilized the activity of this enzyme for more than 72 hr, after which only 25% of the original activity remainded.

In view of these observations, ammonium sulfate precipitate was applied to a Sephadex G-200 column without dialysis. The purification procedure, which involved ammonium sulfate precipitation and gel filtration, yielded *M. gypseum* enzyme that was ca. 33-fold purified (Table 2). Similar observations have been reported for the *E. coli* enzyme, which was purified to homogeneity (4). We noted an enzyme recovery of 225% in this study, which was relatively high compared with that reported for the *E. coli* enzyme (10). The observed high recovery of *M. gypseum* enzyme in our study might be caused by activation of the enzyme as it occurred only when the enzyme was precipitated with ammonium sulfate. Our further experiments to check the effect of ammonium sulfate on glycerol kinase also supported the above activation phenomenon.

M. gypseum glycerol kinase showed 2 pH optima of 8.0 and 10.5 (Fig. 2). This property has not been reported for glycerol kinase from

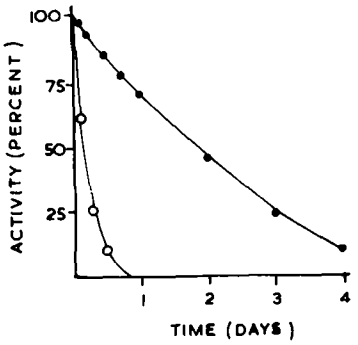

FIG. 1. Effect of ammonium sulfate on enzyme activity • — •; 400 mM ammonium sulfate was added to the homogenate maintained at 4 C; ○— ○, ammonium sulfate. Samples were removed at times indicated and assayed for glycerol kinase.

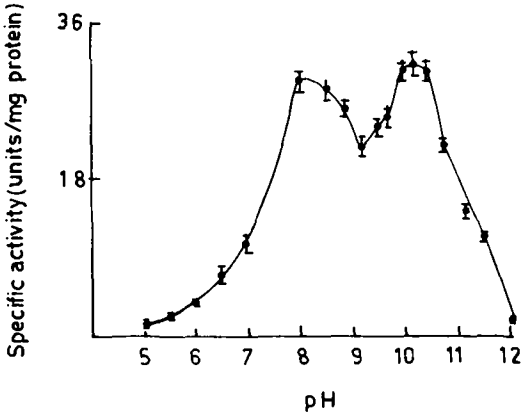

FIG. 2. pH Optima for the purified glycerol kinase of *M. gypseum.* Buffers of Tris-maleate (pH 5.0-7.0), Tris-HCl (pH 7.0-9.0) and glycine-NaOH (pH 9.0-12.0) were used. Values mean ± SD of 4 independent determinations.

TABLE 2

Purification Procedure of Glycerol Kinase from *M. gypseum*

Purification step	Protein (mg)	Total activity (units)	Specific activity (units)	Recovery yield (%)	Purification (fold)
Crude	383.6	5485.4	14.3	100.0	1.0
105,000 × *g* supernatant	233.7	5421.8	23.2	98.8	1.62
Ammonium sulfate (60%) precipitate	85.5	12226.5	143.1	222.8	10.0
Sephadex G-200	25.7	12319.5	478.8	224.5	33.4

other sources (2-4). The molecular weight (4.5×10^5) of the enzyme, determined by gel filtration on Sephadex G-200, is considerably higher than that reported for glycerol kinase of *E. coli* (4). As expected for kinases, Mg^{2+} is required for the enzyme activity (Table 3) and Co^{2+} could replace Mg^{2+} to a limited extent, whereas in *E. coli* (4), Mn^{2+} was reported to be a substitute for Mg^{2+}. Sulfhydryl reagents did not activate the enzyme and iodoacetate did not inhibit the enzyme of *M. gypseum*. This suggested that sulfhydryl groups are not required. However, enzyme from other sources required sulfhydryl groups for their activity (2-4). All tested nucleotides acted as substrate for glycerol kinase of *M. gypseum* (Table 3), unlike the *E. coli* enzyme, which exhibited specificity only for ATP (4).

Double reciprocal plots of initial velocity vs ATP or glycerol concentrations at various fixed concentrations of either glycerol or ATP are presented in Fig. 3 and 4, respectively. A set of paralle lines were obtained in both studies, as expected for an enzyme that catalyzes a 2 substrate reaction via a ping-pong mechanism (13), whereas an ordered sequential mechanism was reported for the *C. mycoderma* enzyme (14). Km values of 2.3 mM and 0.35 mM were obtained from Figures 3 and 4. Double reciprocal plots of initial velocity against the concentrations of glycerol in the absence and presence of fructose 1,6-bisphosphate (Fig. 5) gave a set of lines that intersect on the ordinate. Similar results were also observed with glucose 6-phosphate (data not presented), showing competitive inhibition of the enzyme by these compounds. Glucose, which is an end product of

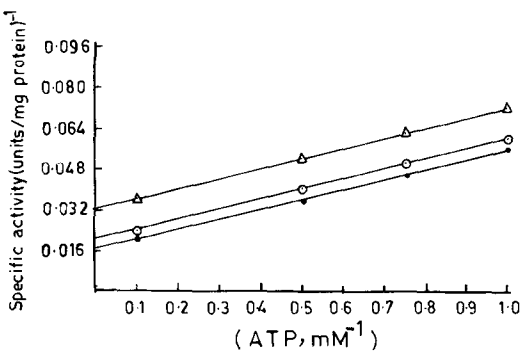

FIG. 3. Double reciprocal plots of initial velocity vs ATP concentration at various fixed glycerol concentrations. Symbols: △——△, 0.05 mM; ○——○, 0.25 mM; ●——●, 2.5 mM. Least squares were used to draw the lines.

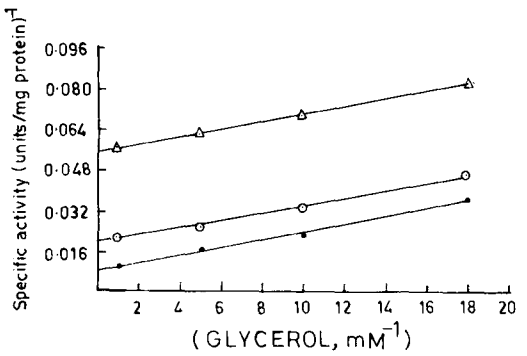

FIG. 4. Double reciprocal plots of initial velocity vs glycerol concentration at various fixed ATP concentrations. Symbols: △——△, 1 mM; ○——○, 5 mM; ●——●, 10 mM. Least squares were used to draw the lines.

TABLE 3

Requirement of Nucleotides and Metal Ions for *M. gypseum* Glycerol Kinase Activity

Nucleotide (10 mM) Metal ions (8 mM)	Specific activity (units/mg protein)
ATP	11.5 ± 1.50
TTP	4.4 ± 1.1
GTP	2.0 ± 0.05
ITP	1.1 ± 0.05
CTP	1.2 ± 0.05
UTP	1.1 ± 0.15
Mg^{2+}	28.6 ± 0.83
Co^{2+}	5.2 ± 0.13
Mn^{2+}	1.92 ± 0.22
Cu^{2+}	–
Ba^{2+}	–

Values are mean ± SD of 4 independent determinations.

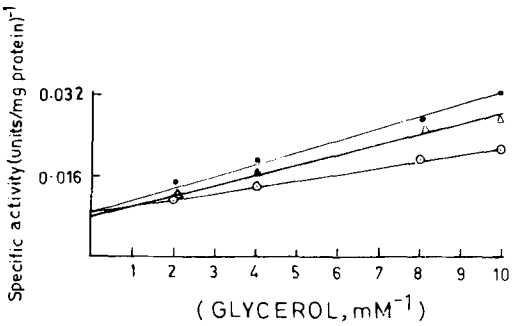

FIG. 5. Competitive inhibition of purified *M. gypseum* glycerol kinase by fructose, 1,6-diphosphate. Double reciprocal plot of initial velocity vs glycerol concentration in the absence (○——○) or presence of 5 mM (●——●) or 2.5 mM (△——△) fructose 1, 6-bisphosphate. Least squares were used to draw the lines.

gluconeogenesis from glycerol, had no effect on enzyme activity. This observed influence of glycerol kinase activity by these glycolysis intermediates suggests the role of phosphate groups in the regulation of glycerol 3-phosphate synthesis. The kinetic properties of this partially purified glycerol kinase is subject to change when purified to homogeneity.

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REFERENCES

1. Bansal, V.S., Talwar, P., and Khuller, G.K. (1982) IRCS Med. Sci. 10, 335.
2. Bublitz, C., and Kennedy, E.P. (1954) J. Biol. Chem. 211, 951-961.
3. Bublitz, C., and Weiland, O. (1962) Methods Enzymol. V, 354-361.
4. Hayashi, S.I., and Lin, E.C.C. (1967) J. Biol. Chem. 242, 1030-1035.
5. Chopra, A., and Khuller, G.K. (1983) Prog. Lipid Res. 22, 189-220.
6. Kasinathan, C., and Khuller, G.K. (1983) Biochim. Biophys. Acta 752, 187-191.
7. Kasinathan, C., Chopra, A., and Khuller, G.K. (1982) Lipids 17, 859-863.
8. Kasinathan, C., Govindarajan, U., Chopra, A., and Khuller, G.K. (1983) Lipids 18, 727-731.
9. Bansal, V.S., Chopra, A., Kasinathan, C., and Khuller, G.K. (1982) Indian J. Med. Res. 76: 832-836.
10. Khuller, G.K., Kasinathan, C., Bansal, V.S., and Chopra, A. (1981) Ind. J. Exp. Biol. 19, 1054-1057.
11. Lowry, O.H., Rosenbrough, N.J., Farr, A., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-276.
12. Jungries, A.M., Dailey, J.N., and Hearth, M.L. (1975) Am. J. Physiol. 229, 1448-1454.
13. Cleland, W.W. (1964) Biochim. Biophys. Acta 67, 104-137.
14. Cheryl, A.J., and Cleland, W.W. (1974) J. Biol. Chem. 249, 2562-2566.

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Linoleate Hydroperoxides Are Cleaved Heterolytically into Aldehydes by a Lewis Acid in Aprotic Solvent

H.W. GARDNER and **R.D. PLATTNER**, *Northern Regional Research Center, Agricultural Research Service, USDA,*[2] *Peoria, IL 61604*

ABSTRACT

Treatment of isomeric methyl linoleate hydroperoxides with a Lewis acid, BF_3, in anhydrous ether led to a carbon-to-oxygen rearrangement that caused cleavage into shorter-chain aldehydes. Methyl (9*Z*,11*E*)-13-hydroperoxy-9,11-octadecadienoate afforded mainly hexanal and methyl (*E*)-12-oxo-10-dodecenoate, whereas methyl (10*E*,12*Z*)-9-hydroperoxy-10,12-octadecadienoate cleaved into 2-nonenal and methyl 9-oxononanoate. The 2 aldehydes obtained from each hydroperoxide isomer were uncharacteristic of the complex volatile profile usually obtained by β-scission of oxy radicals derived from homolysis of the hydroperoxide group. Rather, the reaction resembled the one catalyzed by the plant enzyme, hydroperoxide lyase.

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INTRODUCTION

The acid-catalyzed, carbon-to-oxygen rearrangement of organic hydroperoxides has been known for over a half century (1,2). Because the migratory aptitude for rearrangement is greater for groups able to sustain a positive charge, aryl or vinyl groups rearrange in preference to H or alkyl moieties (1), as illustrated by the well-known rearrangement of cumene hydroperoxide to phenol and acetone.

Application of this rearrangement to fatty hydroperoxides predicts a similar cleavage into shorter-chain aldehydes (Fig. 1). Thus, treatment of autoxidized triglycerides of fatty esters with HCl-treated earths, e.g., Fuller's earth, bleaching earth or celite, caused increased production of volatile aldehydes (3-7). Kimoto and Gaddis (7) reported that HCl-treated Fuller's earth decomposed autoxidized trilinolein into a greater yield and more selective distribution of volatile aldehydes than those obtained from treatment of the autoxidized trilinolein with a free-radical catalyst. They further showed that the volatiles were mainly hexanal and nonenal in the conspicuous absence of dienals. These results may be explained by applying the mechanism of Figure 1 to a mixture of 9- and 13-hydroperoxide isomers in the autoxidized trilinolein. More recently, Grosch et al. (8) treated pure isomers of either linoleic acid hydroperoxide or linolenic acid hydroperoxide with trichloroacetic acid in benzene. From (9*Z*,11*E*)-13-hydroperoxy-9,11-octadecadienoic acid they obtained hexanal, and from (10*E*,12*Z*)-9-hydroperoxy-10,12-octadecadienoic acid, both 2-nonenal and hexanal were identified. By contrast, Gardner et al. (9) found that treatment of (9*Z*,11*E*)-13-hydroperoxy-9,11-octadecadienoic acid with H_2SO_4 in protic solvent gave mostly intramolecular cyclization of the

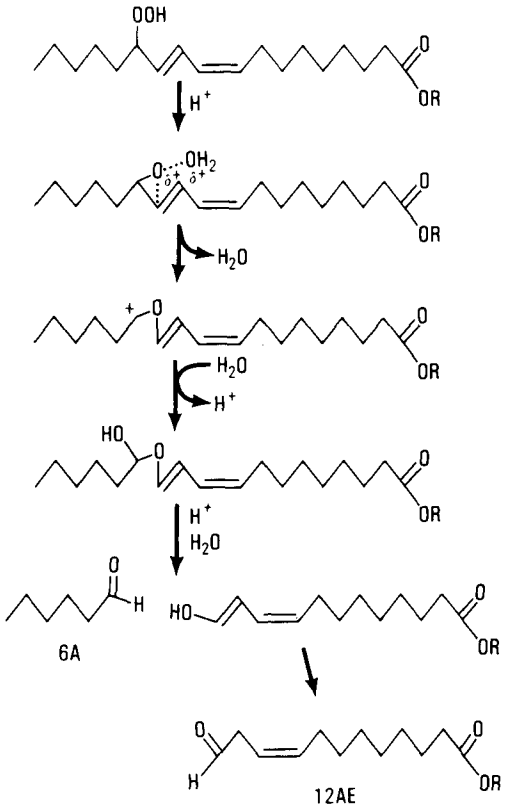

FIG. 1. Predicted mechanism for acid-catalyzed rearrangement of (9*Z*,11*E*)-13-hydroperoxy-9,11-octadecadienoate.

[1] Presented in part at the American Oil Chemists' Society Meeting, Chicago, Illinois, May 8-12, 1983.

[2] The mention of firm names or trade products does not imply that they are endorsed or recommended by the USDA over other firms or similar products not mentioned.

hydroperoxide into 12,13-epoxides with very little cleavage into aldehydes.

Using a Lewis acid (BF_3) in aprotic solvent (anhydrous ether), we report here a much enhanced cleavage of methyl linoleate hydroperoxides than with the result obtained with protic systems (9). The use of rigorously purified hydroperoxide isomers, either the 9- or 13-hydroperoxide of methyl linoleate, and complete analyses of cleavage products allowed a more comprehensive assessment of this acid-catalyzed rearrangement.

METHODS

Preparation of Hydroperoxides

(9*Z*,11*E*)-13-Hydroperoxy-9,11-octadecadienoic acid was prepared by soy lipoxygenase oxidation of linoleic acid, followed by chromatography of the oxidation product through a silicic acid column (10). The isolate (ca. 99+% pure) was esterified with diazomethane, and the ester either was used directly for a large-scale (89 mg) reaction or purified further in smaller quantities by high performance liquid chromatography (HPLC). For HPLC, samples of the 13-hydroperoxide (0.5 mg) were applied to a 9.4 × 250 mm column packed with 10 μm silicic acid (Whatman) and eluted with 5% acetone in hexane (v/v) at a flow rate of 2 ml/min. The 13-hydroperoxide isomer eluted as the main peak after ca. 49 min.

(10*E*,12*Z*)-9-Hydroperoxy-10,12-octadecadienoic acid was prepared by tomato lipoxygenase oxidation of linoleic acid (11), followed by silicic acid column chromatography of the oxidation mixture (10). The isolate from column chromatography (ca. 97%) was esterified with diazomethane and separated further by HPLC. Although the esterified isolate from the silicic acid column was largely methyl (10*E*,12*Z*)-9-hydroperoxy-10,12-octadecadienoate, the sample was contaminated with a few percent of other isomeric hydroperoxides determined by HPLC. These contaminating esters were particularly problematic as they could not be easily separated from the hydroperoxide of interest by silicic acid HPLC because of peak overlap (12). Thus, we used RP-HPLC to effect a preliminary separation of (*E*,*Z*)- from (*E*,*E*)-dienes. Samples (1 mg) were applied to a Whatman C-18 HPLC column (Partisil M-9, 10/50, ODS-2, 9.4 × 500 mm) and eluted with methanol/H_2O (75:25) at 4 ml/min. The fastest eluting peak (96 min) contained the (*E*,*Z*)-dienes. During subsequent stages in the isolation, difficulties were encountered with rearrangement between the hydroperoxide isomers similar to that observed by Chan et al. (13); thus, the eluant from the C-18 HPLC column was collected into a solution of 2,2,5,7,8-pentamethyl-6-hydroxychroman (an α-tocopherol model). The amount of α-tocopherol model compound used was 1/10-1/25 by weight of the hydroperoxide isolated. The antioxidant was carried through the sample manipulations until the last step, i.e., final purification by silicic acid HPLC. Presumably, the antioxidant suppressed peroxy radical formation, which is known to cause hydroperoxide isomer rearrangement (13). Finally, separation by silicic acid HPLC isolated methyl (10*E*,12*Z*)-9-hydroperoxy-10,12-octadecadienoate in high purity from the antioxidant and other remaining impurities. The silicic acid HPLC method was the same as given above for the 13-hydroperoxide. At 2.7 ml/min solvent flow, the compound of interest eluted after ca. 46 min.

All the isolated methyl linoleate hydroperoxide samples were quantitated by their UV absorption at 233 nm using a molar absorptivity of 24,500 (14).

Reaction of Hydroperoxides with BF_3-Ether

All hydroperoxide samples were reacted at concentrations ranging between 2.25-2.8 mM in BF_3-ether solution. The BF_3-ether solution was 0.5% by volume BF_3-etherate (Eastman-Kodak, Rochester, NY, redistilled in a closed system) in anhydrous ether (redistilled from $LiAlH_4$). The hydroperoxides were reacted with the BF_3-ether solution for 5 min at 25 C, and then quenched with an equal volume of water. The ether layer was then washed 3 times with a volume of water 1/5 that of the ether layer. TLC (Silica Gel G, hexane/ether [3:2]) and HPLC of the products revealed that conversion of the hydroperoxide was 90-95% complete.

A large-scale reaction was used exclusively for HPLC isolation of methyl (*E*)-12-oxo-10-dodecenoate for analyses by nuclear magnetic resonance spectroscopy (NMR) and infrared spectroscopy (IR). To this end, 89 mg methyl (9*Z*,11*E*)-13-hydroperoxy-9,11-octadecadienoate (2.8 mM) was reacted with the BF_3-ether solution.

For capillary gas chromatography-mass spectrometry (GC-MS), HPLC-isolated methyl linoleate hydroperoxides (300-400-μg) were spiked with a measured quantity of internal standard, methyl stearate, equivalent to 10-11% by weight of the hydroperoxide sample (see Fig. 2 for specific quantities). Both the hydroperoxide and methyl stearate were then treated with BF_3-ether. The samples were analyzed by GC-MS directly from the water-washed BF_3-ether solution. As expected, treatment with

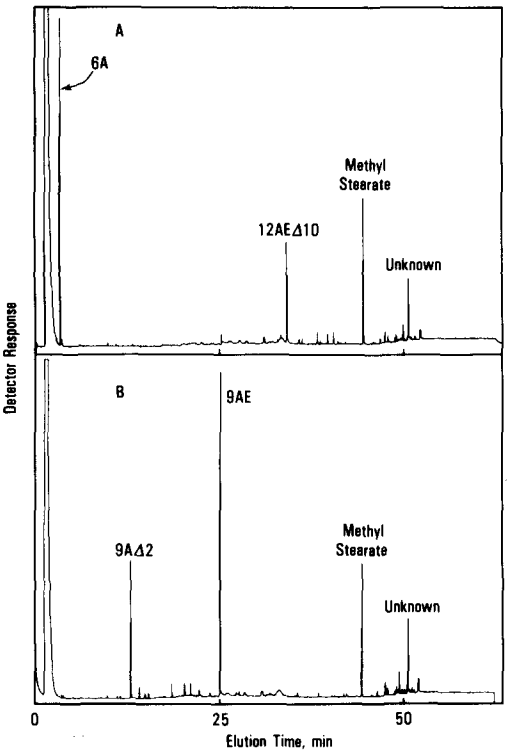

FIG. 2. Capillary GC-MS of products from BF_3-ether treated methyl linoleate hydroperoxides. Peak response is total ionization (EI). (A) Methyl (9*Z*,11*E*)-13-hydroperoxy-9,11-octadecadienoate (387 μg) was treated with 500 μl BF_3-ether solution with 40 μg methyl stearate serving as an internal standard. (B) Methyl (10*E*,12*Z*)-9-hydroperoxy-10,12-octadecadienoate (367 μg) was treated with 500 μl BF_3-ether solution with 40 μg methyl stearate serving as an internal standard. Abbreviations: 6A, hexanal; 12AE-Δ10, methyl (E)-12-oxo-10-dodecenoate; 9AΔ2, 2-nonenal; 9AE, methyl 9-oxononanoate.

BF_3-ether solution did not hydrolyze the methyl ester derivatives as ascertained by reacting the product mixture with diazomethane. No difference in the GC-MS profile was observed before and after treatment with diazomethane.

Capillary GC-MS Methods

Capillary GC-MS analyses of hydroperoxides treated with BF_3-ether were performed using a Finnigan 4535/TSQ MS operated as a conventional GC-MS by setting Q_1 and Q_2 in the all-pass mode. Samples were introduced into the MS from a directly coupled 30 m × 0.26 mm fused silica capillary GC column (J and W Scientific, Rancho Cordova, CA). The liquid phase was SE-54 with a film thickness of 0.25 μm. A 10 lb psi was maintained at the head of the column, giving an estimated flow of 0.7 ml/min. Samples were split into a 50:1 ratio. After injection, the column was held at 50 C for 5 min, then programmed at 4 C/min to 250 C and finally held at 250 C until the end of the analysis. Either electron impact spectra (EI) at 70 eV or chemical ionization spectra (CI) with isobutane (0.25 torr) were recorded at a 1 sec scan rate. The EI and CI ions were collected only with m/z greater than 40 and 100, respectively.

In experiments with heat-induced scission products, the hydroperoxides were decomposed in the GC injection port (220 C), and subsequently the volatiles were separated and analyzed by GC-MS. Thus, methyl (9*Z*,11*E*)-13-hydroperoxy-9,11-octadecadienoate (66 μg in 2 μl CS_2) was injected, held for 30 sec before starting the gas flow and then swept into a 15 m × 0.26 mm capillary column (DB-5 from J and W Scientific). The column was held at 20 C for 5 min, programmed to 250 C at 5 C/min and then held at 250 C. The GC-column effluent was split to both an FID and a Kratos MS-30 MS using an ionizing voltage of 70 eV.

Product Isolation and Identification

Hexanal and methyl 9-oxononanoate were identified by comparing their capillary GC retention times and MS with those of authentic standards. Authentic hexanal was obtained from Aldrich Chemical Co., Milwaukee, WI. Methyl 9-oxononanoate and nonanal were synthesized by periodic acid oxidation (15) of methyl 9,10-dihydroxystearate (Analabs, North Haven, CT).

Methyl (*E*)-12-oxo-10-dodecenoate was isolated by silicic acid HPLC using the column described for isolation of hydroperoxides. This product eluted after ca. 1 hr using hexane/acetone (98:2) at a flow rate of 3 ml/min. The isolate was characterized by nuclear magnetic resonance (NMR) and infrared (IR) spectroscopy. The ^{1}H-NMR spectrum was recorded with a Bruker WH-90 operating at 90 MHz with the sample dissolved in $CDCl_3$. The IR spectrum was obtained with a Perkin-Elmer Model 621 using CS_2 solutions in 0.1 mm NaCl cells. Equivalent chain lengths (ECL) were obtained from GC retention time plots of standard saturated fatty methyl esters.

RESULTS AND DISCUSSION

Treatment of methyl (9*Z*,11*E*)-13-hydroperoxy-9,11-octadecadienoate with BF_3-ether gave 2 aldehydes from cleavage of the fatty chain (Fig. 2A). The fast eluting peak had

essentially the same retention time and MS as standard hexanal. The major MS ions and relative intensities were: (EI) m/z–100 (0.2%, M^+), 82 (6.6%, M^+-H_2O), 72 (11%, M^+-CO), 71 (4.6%, M^+-CHO), 67 (7.0%), 57 (29%), 56 (49%), 44 (100%), 43 (51%), 41 (83%); (CI) m/z–101 (100%, M+H^+). The next peak, eluting at an ECL of 14.0, was tentatively identified as a methyl 12-oxododecenoate. The following major MS ions were consistent with the proposed structure: (EI) m/z–195 (2.4%, M^+-CH_3O), 194 (1.3%, M^+-CH_3OH), 166 [3.9%, M^+-(CH_3O+CHO)], 150 (3.4%), 124 (5.8%), 109 (5.5%), 98 (14%), 87 (16%), 83 (23%), 81 (25%), 74 (30%), 69 (29%), 67 (27%), 59 (21%), 55 (74%), 44 (38%), 41 (100%); (CI) m/z–227 (100%, M+H^+), 195 (41%, M^+-CH_3OH). HPLC isolation of this compound and subsequent analyses by ^{1}H-NMR and IR led to the conclusion that it was methyl (*E*)-12-oxo-10-dodecenoate, as demonstrated by the following spectral data: IR (CS_2) 2,720 w (CHO), 1,695 s (CHO), 1,640 m (CH=CH-CHO), 973 m (*E* olefin) cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 9.5 (d, 1, J=7.5 Hz, H12), 6.85 (dt, 1, J=16, 7 Hz, H10), 6.11 (dd, 1, J=16, 7.5 Hz, H11), 2.31 (t, 4, H2, H9). The ^{1}H-NMR coupling of the aldehyde H12 proton with the H11 olefin and the IR absorption at 1,640 cm^{-1} established the position of the double bond at C10,11. The *E*-olefin was readily shown by the ^{1}H-NMR coupling of H10 and H11 (J=16 Hz) and by the IR absorption at 973 cm^{-1}.

BF_3-ether treatment of methyl (10*E*,12*Z*)-9-hydroperoxy-10,12-octadecadienoate produced both 2-nonenal and methyl 9-oxononanoate (Fig. 2B). Methyl 9-oxononanoate was identified by comparing GC retention time (ECL = 11.2) and MS with an authentic standard. The major MS fragment ions were as follows: (EI) m/z–158 (4.3%, M^+-CO), 155 (8.8%, M^+-CH_3O), 143 (15%), 111 (32%), 87 (51%), 83 (39%), 74 (89%), 69 (28%), 67 (25%), 59 (37%), 55 (87%), 43 (86%), 41 (100%); (CI) m/z–187 (100%, M+H^+), 155 (66%, M^+-CH_3O). The 2-nonenal peak (ECL = 7.7) eluted close to authentic nonanal (ECL = 7.8), and produced the following MS data: (EI) m/z–140 (0.1%, M^+), 122 (1.0%, M^+-H_2O), 111 (1.7%, M^+-CHO), 98 (5.6%), 97 (5.1%), 96 (9.1%), 84 (23%), 83 (20%), 81 (11%), 70 (12%), 69 (38%), 67 (13%), 55 (58%), 43 (32%), 41 (100%); (CI) m/z–141 (81%, M+H^+), 123 [100%, M^+-(H+H_2O)]. Although the MS data were indicative of 2-nonenal, no data were obtained on the (*E*) vs (*Z*) configuration of the double bond.

In accord with the theory (Fig. 1), 2, 9-carbon fragments were obtained from the 9-hydroperoxide, and from the 13-hydroperoxide both 6-carbon and 12-carbon aldehydes formed. The intramolecular insertion of a positively charged oxygen atom, which precedes the cleavage, is a fairly common transformation of organic hydroperoxides (1,2), and the present study represents another example of such a rearrangement. The mechanism (Fig. 1) presupposes that hexanal and methyl (*Z*)-12-oxo-9-dodecenoate forms from the 13-hydroperoxide, but instead hexanal and methyl (*E*)-12-oxo-10-dodecenoate were isolated. Because BF_3 is one of the strongest Lewis acids known, this result was not unexpected, as the conversion of (*Z*)-β,γ-unsaturated carbonyls to (*E*)-α,β-unsaturated carbonyls generally occurs in acid. Likewise, BF_3-ether cleavage of the 9-hydroperoxide would be expected to give (*E*)-2-nonenal instead of (*Z*)-3-nonenal, but the configuration of the double bond was not directly demonstrated.

In Figure 1, the participation of H_2O in the reaction is implied; however, we demonstrated in this study that the cleavage occurred in an aprotic system. BF_3, instead of H^+, causes removal of OH^- from the hydroperoxide during oxygen insertion. The carbonium ion thus formed probably reacts with a stoichiometric amount of released hydroxyfluoroborate ion $[BF_3OH]^-$ (or adventitious H_2O) to form a hemiacetal. As in the mutarotation of sugars, hydrolysis of hemiacetal to free aldehydes requires an acid as well as H_2O, but H_2O plays only a catalytic role (16). Because a strong aldehyde odor was noted before the reaction was terminated, adventitious H_2O or hydroxyfluoroborate probably was sufficient to cause hydrolysis of the hemiacetal; however, quenching the reaction with H_2O to terminate the process may have aided hydrolysis to some extent. The proposed hemiacetal intermediate may be identical to the "unknown" peaks eluting at later times in the GLC elution (Fig. 2). Inspection of the CI-MS of the unknown from Figure 2A (13-hydroperoxide product) revealed all the ions expected for a CI-MS of a mixture of hexanal and methyl (E)-12-oxo-10-dodecenoate, indicating that the structure of the unknown fragmented readily into these aldehydes under CI-MS conditions. A weak m/z 309 ion (4.4%) was the only other ion present in the spectrum, and this fragment ion was consistent with the carbonium ion shown in Figure 1. The facile loss of hydroxyl from the hemiacetal (or H_2O from M+H^+) during CI-MS does not seem unreasonable. Likewise, a CI-MS of the unknown from Figure 2B (9-hydroperoxide product) afforded all the ions expected of a mixture of methyl 9-oxononanoate and 2-nonenal, and the only other significant ion was m/z 309

(7.1%). A very small ion at m/z 325 may have indicated the presence of a M-H$^+$ ion.

The present report extends earlier research in several ways. In previous investigations (3-8), the cleavage fragments from the acyl portion of the fatty chain were not examined, but the present work now reports these fragments. Moreover, in early studies (3-7) the fatty hydroperoxides were mixtures of positional isomers obtained from autoxidation, and thus, a mixture of cleavage products were obtained. The observation of hexanal and nonenal in the latter studies is consistent with the results presented here using pure positional isomers. Also, this communication generally agrees with the recent report of Grosch et al. (8), who used specific isomers of the hydroperoxides, but a major departure of their data from ours regards their observation of a 3.8:1 ratio of 2-nonenal to hexanal from the 9-hydroperoxide of linoleic acid. Because they did not report the actual purity of their sample of 9-hydroperoxide, surmising the cause of hexanal formation is difficult. Grosch et al. (8) obtained a low yield of volatile aldehydes (1.5-6.9 mol%) from hydroperoxides by the use of a protic acid (trichloroacetic acid) in aprotic solvent (benzene), and thus, other factors may have contributed to their result. In protic solvent (CH_3OH/H_2O) with protic acid (H_2SO_4), cleavage of the fatty chain was very minor, but instead, epoxymethoxyoctadecenoic and epoxyhydroxyoctadecenoic acids were major products of linoleic acid hydroperoxide (9). Under these conditions, oxygen rearrangement of the 13-hydroperoxide of linoleic acid mainly resulted in 12,13-epoxides, and additional substitution from solvent at either C-9 or C-11 was indicative of a transitional epoxyallylic cation with the positive charge localized between C-9 and C-11. In the aprotic reaction reported in the present communication, the positive charge in the transition state is proposed to reside instead at C-13 (Fig. 1). This subtle difference in mechanism between the aprotic and protic reactions is of theoretical interest.

The BF_3-ether cleavage of fatty hydroperoxides resembled the reaction catalyzed by the plant enzyme, hydroperoxide lyase (17). This acid-catalyzed heterolysis may indicate a mechanism for the enzymic process. The function of hydroperoxide lyase in plants is not fully understood, but a few studies have indicated that the enzyme may be involved as a plant defense mechanism. For example, (*E*)-12-oxo-10-dodecenoic acid, which is isomerized from the lyase product, (*Z*)-12-oxo-9-dodecenoic acid, responded like a wound hormone, and thus, was named "traumatin" by Zimmerman and Coudron (18). Also, 2-hexenal, which is readily derived from the lyase product, (*Z*)-3-hexenal, has been reported to be a potent inhibitor of fungal growth in leaves (19). The BF_3-ether cleavage of hydroperoxides reported here may offer a facile route to some of the hydroperoxide lyase products for further studies of their biological significance in plants.

The acid-catalyzed cleavage to aldehydes does not fully resemble the scission encountered with the free-radical decomposition of fatty hydroperoxides. β-Scission of the oxy radical, derived from homolysis of methyl (9*Z*, 11*E*)-13-hydroperoxy-9,11-octadecadienoate, predicts pentane and methyl 13-oxo-9,11-tridecadienoate from a C-13,14 cleavage (Fig. 3). In actual experiments of the heat decomposition of the 13-hydroperoxide (supposedly a free-radical process), pentane and methyl (9*Z*,11*E*)- and (9*E*,11*E*)-13-oxo-9,11-tridecadienoates were produced readily (Fig. 4). Pentane was identified from library MS after the spectrum was subtracted because of the CS_2 solvent. The 2 isomeric methyl 13-oxo-9,11-tridecadienoate peaks were identified by their relative GLC retention and their known MS (20). Neither of these fragments were observed after the BF_3-ether reaction. The appearance of minor amounts of methyl octanoate and methyl 9-oxononanoate can be explained by the well-known radical rearrangement of hydroperoxide positional isomers through β-scission of peroxy radicals (21). However, Chan et al. (21) reported greatly exaggerated molar ratios

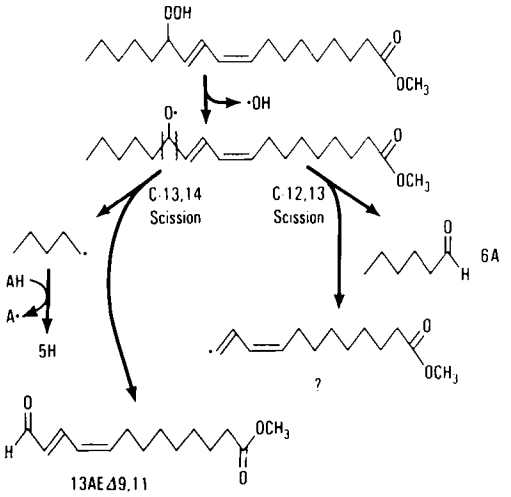

FIG. 3. Generally accepted mechanism for chain scission of methyl (9*Z*,11*E*)-13-hydroperoxy-9,11-octadecadienoate via an oxy radical. The question mark denotes a vinyl radical, which should be energetically disfavored.

for methyl octanoate and methyl 9-oxononanoate from the decomposition of the 13-hydroperoxide, because they failed to analyze the relatively large amount of methyl 13-oxo-9,11-tridecadienoates demonstrated in our present communication.

Free-radical scission of the C-13,14 bond is undoubtedly important, but radical scission of the C-12,13 bond creating hexanal and a C-12 vinyl radical (Fig. 3) should not be favored because of energetic arguments against it (22). That is, the heat of formation, as well as the related bond dissociation energy, required for the formation of the vinyl radical is comparatively much larger than for a hydrocarbon radical (pentyl radical). Thus, the formation of hexanal observed in Figure 4 presented an enigma. Does hexanal originate via a free-radical scission of C-12,13 (23), or is it generated by heat-accelerated heterolysis of the hydroperoxide? Isomeric methyl 12-oxododecenoates, which also could be derived from heterolytic cleavage, were observed after heat treatment of the 13-hydroperoxide (Fig. 4). Carbonyls, like methyl 12-oxododecenoate, have been suggested as originating from a combination of the vinyl radical with a hydroxyl radical (23), and a hydroxyl radical from hydroperoxide homolysis conceivably could be trapped by a cage effect for subsequent reaction with the vinyl radical produced by scission. However, in reactions thought to be exclusively radical processes, e.g., the heat decomposition of hydroperoxides, heterolytic mechanisms also may be involved in the scission to volatiles.

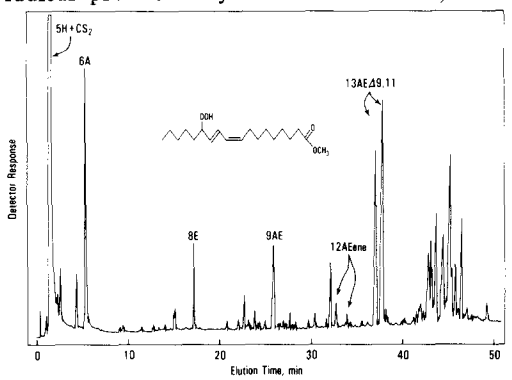

FIG. 4. Capillary GC of heat-induced (220 C) volatiles from decomposition of methyl (9*Z*,11*E*)-13-hydroperoxy-9,11-octadecadienoate. The peak response was obtained by FID. Abbreviations: 5H, pentane; 6A, hexanal; 8E, methyl octanoate; 9AE, methyl 9-oxononanoate; 12AE ene, methyl 12-oxododecenoate isomers (double bond not specified); 13AEΔ9,11,methyl 13-oxo-9,11-tridecadienoate isomers.

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REFERENCES

1. Hiatt, R. (1971) in Organic Peroxides (Swern, D., ed.) Vol. II, pp. 65-70, Interscience, New York.
2. Smith, P.A.S. (1967) in Molecular Rearrangements (de Mayo, P., ed.) Part 1, pp. 568-577, Interscience, New York.
3. Täufel, K., and Heder, G. (1963) Fette Seifen Anstrichm. 65, 85-91.
4. Horikx, M.M. (1964) J. Appl. Chem. 14, 50-52.
5. Ellis, R., Gaddis, A.M., Currie, G.T., and Powell, S.L. (1968) J. Am. Oil Chem. Soc. 45, 553-559.
6. Schwartz, D.P., Haller, H.S., and Keeney, M. (1963) Anal. Chem. 35, 2191-2194.
7. Kimoto, W.I., and Gaddis, A.M. (1969) J. Am. Oil Chem. Soc. 46, 403-408.
8. Grosch, W., Schieberle, P., and Laskawy, G. (1981) in Flavour '81 (Schreier, P., ed.) pp. 443-444, Walter de Gruyter, Berlin, Germany.
9. Gardner, H.W., Weisleder, D., and Nelson, E.C. (in press) J. Org. Chem.
10. Gardner, H.W. (1975) Lipids 10, 248-252.
11. Matthew, J.A., Chan, H.W.-S., and Galliard, T. (1977) Lipids 12, 324-326.
12. Chan, H.W.-S., and Levett, G. (1977) Lipids 12, 99-104.
13. Chan, H.W.-S., Levett, G., and Matthew, J.A. (1979) Chem. Phys. Lipids 24, 245-256.
14. Johnston, A.E., Zilch, K.T., Selke, E., and Dutton, H.J. (1961) J. Am. Oil Chem. Soc. 38, 367-371.
15. Kleiman, R., Spencer, G.F., Tjarks, L.W., and Earle, F.R. (1971) Lipids 6, 617-622.
16. Gould, E.S. (1959) in Mechanism and Structure in Organic Chemistry, pp. 541-543, Holt-Dryden, New York.
17. Gardner, H.W. (1980) in Autoxidation in Food and Biological Systems (Simic, M.G., and Karel, M., eds.) pp. 486-489, Plenum, New York.
18. Zimmerman, D.C., and Coudron, C.A. (1979) Plant Physiol. 63, 536-541.
19. Major, R.T., Marchini, P., and Sproston, T. (1960) J. Biol. Chem. 235, 3298-3299.
20. Frankel, E.N., Neff, W.E., and Selke, E. (1983) Lipids 18, 353-357.
21. Chan, H.W.-S., Prescott, F.A.A., and Swoboda, P.A.T. (1976) J. Am. Oil Chem. Soc. 53, 572-576.
22. O'Neal, H.E., and Benson, S.W. (1973) in Free Radicals (Kochi, J.K., ed.) pp. 284-285, Wiley & Sons, New York.
23. Frankel, E.N. (1983) Prog. Lipid Res. 22, 1-33.

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ERRATUM

In The Analysis of Triglyceride Species by High Performance Liquid Chromatography via a Flame Ionization Detector, by F.C. Phillips et al. in the February 1984 issue of *Lipids*, Figures 7 and 8 were mislabeled. The 2 figures, labeled correctly, follow.

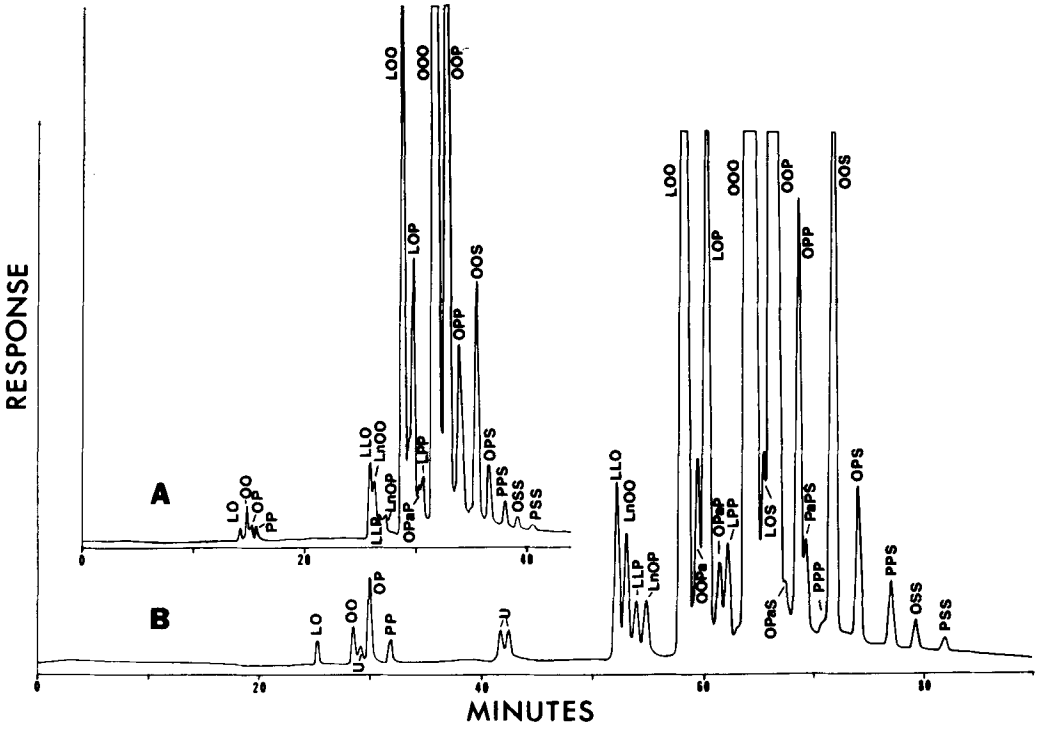

FIG. 7. Triglyceride species analyses of olive oil. A, the conditions are as in Figure 1A except that a gradient from 30 to 60% was used; B, Zorbax columns I and II were connected and a 120 min gradient used. Species identification as indicated where P = palmitate, S = stearate, O = oleate, L = linoleate, Ln = linolenate, Pa = palmitate, S = stearate, O = oleate, L = linoleate, Ln = linolenate, Pa = palmitoleate and U = unidentified. The order of the designations does not indicate the separation of isomers.

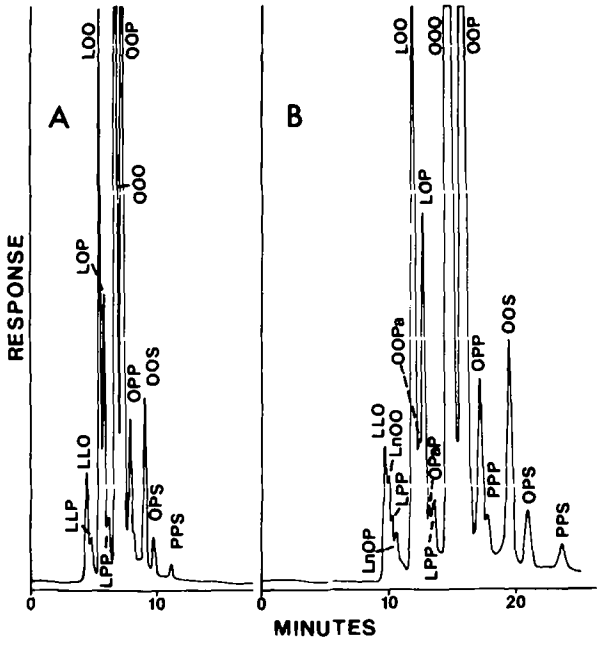

FIG. 8. The triglyceride species analysis of olive oil with Zorbax column III. Isocratic elution with 45% methylene chloride in acetonitrile; sample size 100 μg and FID with a 1:1 effluent split. A = flow rate of 1.2 ml/min and B = 0.6 ml/min.

LETTER TO THE EDITOR

Lipid Contaminants in Commercial Lipases

Sir:

During lipolysis of wax esters on thin layer chromatography (TLC) plates (1), abnormally high errors for stearic (18:0), palmitic (16:0) and oleic (18:1) acids were found in the hydrolysates. These anomalous results prompted us to examine the source of the contaminations, which was found to be the lipase used.

We have analyzed 2 commercial lipase samples that were used for the lipolysis of wax esters and developed a procedure for the preparation of commercial lipase free of fatty acid (FA) contaminants. Pancreatic lipase from 300 mg each from Sigma Chemical Co. (St. Louis, MO, Type II, crude from porcine pancreas) and from Calbiochem (Los Angeles, CA, B grade from porcine pancreas) were mixed vigorously with 15 ml of redistilled diethyl ether and centrifuged. The supernatnats were carefully removed with a syringe and saved. The extraction procedure with diethyl ether was repeated 6 times for each lipase sample. The pooled etherial extracts were dried over anhydrous sodium sulphate and the solvent was evaporated and weighed. The lipases were then washed 6 times with 15 ml protions of acetone. The acetone extracts were also pooled and the solvent was evaporated and weighed.

The etherial extracts were spotted on preparative TLC plates that were developed using a solvent system of light petroleum ether (40 C-60 C) diethyl ether/acetic acid (80:20:1.5, v/v). The bands were visualized by putting the plates in an iodine chamber. The bands were identified by comparing the R_f values with those of known standards. Finally, the various bands were scraped off the plates and the compounds were extracted using diethyl ether, the solvent was evaporated and then weighed. The free fatty acid (FFA) bands from the 2 lipase samples were methylated using diazomethane (2). To each of the methyl ester samples, 50 μg of methyl pentadecanoate (15:0) were added and analyzed by gas liquid chromatography (GLC) using a 10% DEGS column. From the chromatograms, peak areas were determined and each of the components was identified and quantified.

Beeswax and *Avicennia officinalis* leafwax were hydrolyzed with extracted and unextracted lipases on TLC plates, using 1 mg lipase and 1 mg wax esters, according to the method described by Misra et al. (1). After developing the plates, the FA bands were scraped off the plates, extracted and methylated with diazomethane. To each of the methyl ester samples, 50 μg methyl pentadecanoate were added and analyzed by GLC, using a 10% DEGS column.

Enzyme activities, determined according to Luddy et al. (3), were 111 units for Sigma and 212 units for Calbiochem lipases before the extraction procedures. After extraction with solvents, the activities were found to be 105 and 198 units for Sigma and Calbiochem lipases. Unit activity is defined as: 1.0 μ equivalent of oleic acid hydrolyzed from triolein in 1 hr at pH 7.7 and 37 C.

The percentages of ether-extracted lipids in Sigma and Calbiochem samples were 5.1% and 4.16% of the lipases. Acetone-extracted lipids in Sigma (4.2%) and Calbiochem (0.4%) indicate that ether-extractable lipids were present in the highest proportion. Table 1 shows that major contaminants in both the commercial lipases were FA. The compositions of FA are presented in Table 2. The major contaminant in both lipases was 18:0 along with appreciable amounts of 16:0 and 18:1, with other minor components. The FA compositions of beeswax and *A. officinalis* leaf wax after lipolysis with extracted lipases are also presented in Table 2. The FA compositions of beeswax and *A. officinalis* leaf wax after incubation with extracted and unextracted lipases are presented in Table 3. Table 3 shows that considerable error exists in the compositions of 16:0, 18:0 and 18:1 when unextracted lipases were used but both solvent extracted lipases produced results identical to those by chemical hydrolysis of the waxes (1).

From the results of the present study, considerable contamination is in the FA pool when commercial pancreatic lipases are used, particularly when the enzyme/substrate ratio is 1:1 and FA analysis of the hydrolysate is required. Consistent results may be obtained by solvent extraction of the lipases before lipolysis, as has been described in this communication.

SUNITI MISRA and A. CHOUDHURY
Department of Marine Science
University of Calcutta
35 B.C. Road
Calcutta 700 019, India

*To whom correspondence should be addressed.

TABLE 1

Compositions (percentage w/w) of Various Neutral Lipid Components in the Ether Extracts of Sigma and Calbiochem Lipases

Components	Sigma lipase		Calbiochem lipase	
	Percentage of ether extract	Percentage of lipase	Percentage of ether extract	Percentage of lipase
Hydrocarbon	3.0	0.15	3.0	0.11
Sterol ester	4.0	0.20	7.0	0.30
Triglyceride	1.0	0.05	5.0	0.20
Fatty acid	90.0	4.60	78.0	3.25
Sterol	2.0	0.10	7.0	0.30
Acetone extract	–	4.20	–	0.40

TABLE 2

Fatty Acid Compositions (μg/mg) of Lipases, Beeswax and *A. officinalis* Leaf Wax

Component acids	Sigma lipase	Calbiochem lipase	Beeswax[a]	*A. officinalis*[a] leaf wax
12:0	0.10	–	–	0.9
13:0	0.05	–	–	–
14:0	0.60	0.5	3.8	3.0
16:0	4.50	11.8	118.6	81.2
18:0	27.70	17.3	21.1	10.4
18:1	12.80	2.7	5.3	83.0
18:2	–	0.2	1.8	34.2
20:0	–	–	2.5	3.9

[a]Incubated (1) for 96 min with extracted Sigma lipase.

TABLE 3

Fatty Acid Compositions (percentage w/w) of Beeswax and *A. officinalis* leaf wax Obtained by Lipolysis Using Extracted and Unextracted Lipases

Component acids	Beeswax			*A. officinalis* leaf wax		
	Extracted lipase	Unextracted lipase		Extracted lipase	Unextracted lipase	
		Sigma	Calbiochem		Sigma	Calbiochem
12:0	–	0.05	–	0.4	0.40	0.4
13:0	–	0.03	–	–	0.02	–
14:0	2.5	2.20	2.3	1.4	1.40	1.4
16:0	77.5	61.90	70.3	37.5	32.60	37.3
18:0	13.8	24.50	20.7	4.8	14.50	11.1
18:1	3.4	9.10	4.3	38.3	36.60	34.4
18:2	1.2	0.90	1.1	15.8	13.00	13.8
20:0	1.6	1.32	1.3	1.8	1.48	1.6

ASHIS K. DUTTA, J. DUTTA and AMITABHA GHOSH*
Department of Chemistry
Bose Institute
93/1 A.P.C. Road
Calcutta 700 009, India

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REFERENCES

1. Misra, S., Choudhury, A., Dutta, A.K., Ghosh, A., and Dutta, J. (1983) J. Chromatogr. 280, 313-320.
2. Schlenk, H., and Gellerman, J.L. (1960) Anal. Chem. 32, 1412-1414.
3. Luddy, F.E., Barford, R.A., Herb, S.F., Magidman, P., and Riemenschneider, R.W. (1964) J. Am. Oil Chem. Soc. 41, 693-396.

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METHODS

A Rapid Procedure for Screening and Isolating Oxygenated Free Fatty Acid Metabolites from Plants and Natural Waters

VAN A. ROGERS, ROBERT T. VAN ALLER,* GEORGE F. PESSONEY, EDWARD J. WATKINS and HAROLD G. LEGGETT, *Departments of Chemistry and Biology, University of Southern Mississippi, Hattiesburg, MS 39401*

ABSTRACT

A rapid method of isolating a relatively pure fraction of oxygenated fatty acids from plants and natural waters is described. These metabolites were isolated from aqueous extracts using octadecylsilyl silica in a reverse-phase batch extraction method. The extraction method, together with reverse-phase analytical high pressure liquid chromatography (HPLC), was used to establish a routine screening method for the presence of these compounds in a variety of natural sources. A reverse-phase preparative HPLC purification method is also described.
Lipids, 19:304-306, 1984.

INTRODUCTION

In a previous paper (1), the isolation of a large number of algal-inhibiting oxygenated fatty acids (OFA) from *Eleocharis microcarpa* Torr. was described. One metabolite was characterized as a C_{20} cyclic trihydroxy fatty acid similar to the prostaglandin F series. Chromatographic evidence from other aquatic plants and natural waters indicates that these metabolites commonly occur in small quantities.

Procedures have been developed using C_{18} bonded-phase silica (ODS silica) to isolate these components from large volumes of aqueous plant extracts and water, and these procedures are simpler and less time-consuming than liquid-liquid extraction and column chromatography. In view of the importance of these metabolites to allelochemical studies and the potential importance of prostaglandinlike compounds, these procedures may be useful to other investigators.

EXPERIMENTAL METHODS

Materials

Fresh plants and water were collected from ponds located in or near Hattiesburg, MS. The plant material was collected by hand or with a course-tooth rake and washed with water. All collected material was kept in plastic containers with as little air as possible. Plants were either processed immediately or stored at −20 C. Aquatic macrophytes screened for fatty acid derivatives were: *E. microcarpa, Potamogeton* spp. (pond weed), *Thalassia* spp. (turtle grass) and *Ruppia* spp. (widgeon grass). The algae screened included, *Chara* spp., *Ocillatoria* spp. and *Chlorella vulgaris.* Pond waters screened were: 37 l from a pond on the campus of the University of Southern Mississippi, 20 l from a farm pond that had a bloom of bluegreen algae, 20 l from a farm pond containing very little phytoplankton and 50 l from a pond that had a dense growth of *E. microcarpa.*

All solvents used were either reagent or high pressure liquid chromatography (HPLC) grade. Reagent grade solvents were distilled in glass before use. All solvents were mixed volume to volume.

Spectral Methods

Infrared spectra were taken neat on a Perkin Elmer 567 spectrophotometer and ultraviolet spectra were taken, in methanol, with a Varian Cary 17 spectrophotometer.

EXTRACTION METHODS

Previous work (1) reported that of the 43 thin layer chromatography (TLC) fractions isolated from *E. microcarpa,* 33 demonstrated an inhibitory effect on the bluegreen alga *Anabena flos-aquae.* Infrared and ultraviolet spectra, chromatography and chemical characterization indicated the inhibitors to be OFA. In the same study, 1 compound was concluded to have a prostaglandinlike structure; therefore, separation techniques specific for these kinds of compounds were chosen. Powell described a rapid and an efficient procedure using ODS silica for the extraction of prostaglandins from body fluids and tissues (2). The procedure is designed for clinical analysis and makes use of very small (10-15 ml) samples. Powell's proce-

*To whom correspondence should be addressed.

dure was modified so that 0.5-4.0 l of aqueous plant extracts and 20-50 l of pond water could be used.

ODS silica was prepared from 60-200 mesh Baker silica according to the procedure of Bennett et al. (3). After preparation, the ODS silica was pretreated by rinsing first with methanol and then with distilled water.

Aquatic Macrophytes

Five hundred ml portions of cooled, acidified aqueous plant extracts, were decanted slowly from particulate matter onto ca. 150 gm pretreated ODS silica. Each portion of the amber-colored extract was gently swirled with the ODS silica until the colored materials were absorbed. The resulting colorless aqueous phase was discarded.

The ODS silica was then gently swirled with 5, 150 ml portions of ethanol/water (15:85, v/v) to elute polar substances. These ethanol/water portions were discarded. The ODS silica was then gently swirled with 5, 150 ml portions of methyl formate to recover the OFA. The methyl formate layers were decanted from the ODS silica, combined in a separatory funnel and allowed to separate into 2 layers. The bottom layer (ethanol/water) was discarded. The ODS silica can be reused after washing with methanol. The top layer (methyl formate/water) was flash evaporated with a Brinkmann ROTAVAPOR-R until water and oily OFA remained. This mixture was extracted with 3, 100 ml portions of chloroform. The pale yellow chloroform layers were combined, dried with anhydrous sodium sulfate, filtered and the chloroform was flash evaporated. The resulting OFA were weighed and stored in an atmosphere of nitrogen at −20 C in chloroform/methanol (2:1) at a concentration of 30 mg/ml.

Two liters of aqueous extract (from 2 kg plant) typically produced a yield of 125-150 ppm OFAs from *E. microcarpa.*

Pond Water

Pond water was filtered through 200 mesh nylon screen to remove plankton and passed through 100 gm of ODS silica contained in a glass column. Water-jet vacuum applied at the effluent end of the column, was necessary to maintain flow rate at or above 200 ml/min. The ODS silica was then extracted with ethanol/water (15:85) and methyl formate as before. The ethanol/water fraction, in contrast to the plant extracts, contained very little polar material. All pond water analyzed produced 1-1.5 ppm of OFA from the methyl-formate fraction. In contrast, chloroform extraction of pond water, followed by alumina chromatography, typically produced only 0.3 to 0.5 ppm.

Infrared spectra and reverse-phase analytical HPLC showed the methyl-formate fractions to correspond to the chloroform/acetone fraction from column chromatography previously described (1).

HPLC

Analytical HPLC

Reverse-phase analytical HPLC described in Reference 1 was used to screen methyl formate fractions for OFA. Component I was isolated previously and shown to be a C_{20} trihydroxy fatty acid derivative. Component II, the major peak, with a retention time 31.5 min, was present in all but 2 samples. Analytical HPLC of other plant genera produced similar chromatograms. *Potamogeton* showed a very strong peak for component II; in the chromatogram of *Thalassia,* a major peak occurred for component I; *Ruppia* showed a minor peak for I and a major peak for II; and *Najas* showed minor peaks for I and II. The chromatogram of *Chara* provided a major peak for I and II, *Ocillatoria* showed a minor peak for I and *Chlorella* minor peaks for both I and II.

The chromatograms of methyl-formate fractions from pond waters were less complex than those from plants. The chromatogram of the university pond showed a major peak for component II. The farm pond with bluegreen algae showed a minor peak for I and the farm pond with no bluegreen algae showed major peaks for I and II. The pond containing *E. microcarpa* showed major peaks for both I and II.

Preparative HPLC of Component II

Component II, from extracts of *E. microcarpa,* was chosen for purification by preparative HPLC because of its proximity to PGE_1 and PGA_1. Two Alltech Magnum 9 columns (250 × 9.4 mm and 500 × 9.4 mm, packed with 10 mM ODS #3) were connected in series. Flow rates were maintained at 3.8 ml/min. A Laboratory Data Control UV detector set at 280 nm was used to monitor eluted components. Various solvent gradients were evaluated; for example, a gradient of water/methanol (2:3) for 10 min, then a 2% change every 30 sec, showed component II to be a major peak with several shoulders. This portion of the chromatogram is shown in Figure 1a. The best resolution obtained employed a gradient of water/methanol (2:3) for 30 min, then 1% change every min. Figure 1b shows the separation of these minor constituents from component II. Injection of 300 mg OFA produced ca. 2 mg component II as a light yellow oil.

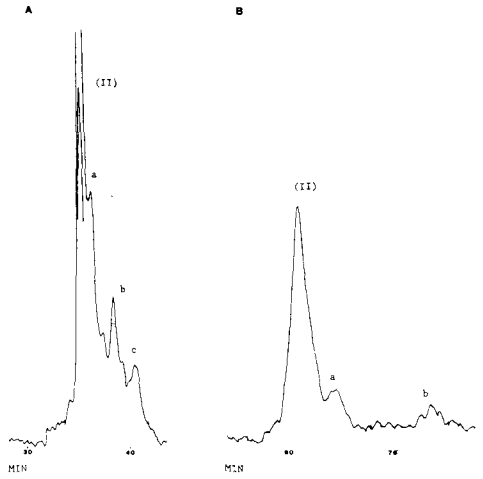

Fig. 1. Preparative HPLC of II.

Comparison of IR, UV and chemical tests indicated II to be in one of the relatively less polar TLC bands reported in the previous paper (1). The analytical HPLC retention time was identical with TLC fraction 6:5. IR (both component II and fraction 6:5) (film) 3,400 cm^{-1} (broad) tailing to 2,300 cm^{-1}, 2,940 cm^{-1} (s), 2,850 cm^{-1} (m), 1,710 cm^{-1} (s), 1,595 cm^{-1} (m). The fingerprint region was also essentially the same. The UV spectrum of the TLC fraction 6:5 showed a major peak at 227 nm and a minor peak at 273 nm. Component II had a major peak at 223 nm and a major peak at 277 nm. Further structural studies are in progress.

DISCUSSION

The methodology described was developed from a clinical procedure so that low concentrations of dissolved organics can be extracted from very large volumes of water rapidly and inexpensively. This approach, when combined with preparative HPLC, can be used to isolate enough pure components for characterization and testing and is not as time-consuming as column and TLC methods. This method is especially suited for use as a rapid screening procedure for these kinds of compounds when smaller batches of plant material are extracted and combined with analytical HPLC.

This method is particularly advantageous for absorbing small quantitites of OFA from natural waters with coarse-mesh ODS silica because flow rates are affected little by fine-particulate matter usually present. Another advantage is that the large quantities of humic and tannic acids present in ponds and streams are not retained on the ODS silica when water is passed through.

REFERENCES

1. van Aller, R.T., Clark, L.R., Pessoney, G.F., Rogers, V.A. (1983) Lipids 18, 617-622.
2. Powell, W.S. (1980) Prostaglandins 20, 947-957.
3. Bennett, H.P.J., Hudson, A.M., McMartin, C., and Purdon, G.E. (1977) Biochem. J. 168, 9.

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COMMUNICATIONS

Thermal Decomposition of Individual Positional Isomers of Methyl Linolenate Hydroperoxides, Hydroperoxy Cyclic Peroxides and Dihydroperoxides

KENNETH E. PEERS*, DAVID T. COXON and **HENRY W.-S. CHAN**, *Agricultural Research Council, Food Research Institute, Colney Lane, Norwich, NR4 7UA, United Kingdom*

ABSTRACT

The methyl esters of 4 individual positional isomers of hydroperoxides, 2 positional isomers of hydroperoxy cyclic peroxides and a 9,16-dihydroperoxide were prepared by autoxidation of methyl linolenate and separated by preparative high pressure liquid chromatography. Isolated hydroperoxide isomers were thermally decomposed and the resulting volatile components analyzed. Each hydroperoxide or hydroperoxy cyclic peroxide isomer yielded characteristic volatile products. The major volatiles from each acyclic hydroperoxide corresponded with those predicted to arise by carbon-carbon scission on either side of the corresponding alkoxy radical intermediate and little evidence was found of isomerization between the various positional isomers occurring during the process. A similar mechanism would account for the volatile products obtained from the cyclic peroxides. 2,3-Pentanedione was a significant odor contributor arising from the 13,15-epidioxy-16-hydroperoxide isomer although it was only a minor decomposition product.
Lipids, 19:307-313, 1984.

INTRODUCTION

The role of hydroperoxides as intermediates in the formation of volatile off flavors from oxidized unsaturated fats is well established (1). The thermal decomposition of unsaturated hydroperoxides is believed to occur via homolytic cleavage of the oxygen-oxygen bond to yield a hydroxyl and an alkoxyl radical. The alkoxyl radical intermediate then decomposes by carbon-carbon cleavage to yield, most commonly, aldehyde or hydrocarbon products. Aldehydes are formed directly but hydrocarbon products result from an alkyl radical that must then abstract a hydrogen atom from an appropriate donor substrate. Alternatively, alkyl radicals may undergo further reactions, e.g., oxidation leading to alcohol or ketone products.

When analyzing volatile products from oxidation of a complex lipid mixture such as a natural vegetable oil or even a simple model system such as methyl linoleate, to know with certainty the origin of many of the volatiles is difficult as with this simple mechanism the expected hydroperoxide precursors do not seem to account for all of the observed products. This failure to account adequately for all of the volatile products could be caused by oversimplified theories for both the formation and decomposition of the hydroperoxides. In the dark, autoxidation of phenyl linoleate in addition to the expected 9- and 13-hydroperoxides, minor amounts of 8-, 10-, 12- and 14-hydroperoxides are produced (2). These products would explain the origin of minor volatiles such as pentanal, 2-heptenal and 2-nonenal from the autoxidation of linoleate. Recent ESR evidence supports the formation of secondary alkyl radicals by hydrogen abstraction from nonallylic chain methylenes in the radical initiated oxidation of both methyl oleate and methyl elaidate (3). Radicals of this type arising during lipid autoxidation would presumably form the corresponding secondary hydroperoxides and provide precursors to a whole range of minor volatiles.

In order to study the decomposition of hydroperoxides to form volatiles, one would ideally wish to use pure single isomer hydroperoxides as reaction substrates. Evans et al. (4) prepared a 13-hydroperoxide of linoleic acid by enzymic oxidation with soybean lipoxygenase and showed that it decomposed under thermal conditions to give pentane as the principal hydrocarbon product. Chan et al. (5) produced individual 9- and 13-hydroperoxides of linoleic acid in high purity (>99%) by enzymic synthesis and high pressure liquid chromatography (HPLC) purification. They obtained the same mixture of volatile decomposition products (hexanal, methyl octanoate, *cis,trans*- and *trans,trans*-2,4-decadienals and methyl 9-oxononanoate) from either isomer, although in different proportions. Frankel et al. (6) carried out an extensive investigation of the volatile thermal decomposition products of hydroperoxide fractions from autoxidation and

*To whom correspondence should be addressed.

photosensitized oxidation of methyl oleate, linoleate and linolenate. Their study did not use individual purified isomers but purified mixtures of isomers. Frankel et al. (7) have also reported on the volatile thermal decomposition products of pure isomeric hydroperoxy cyclic peroxides derived from methyl linolenate.

In this study, we have prepared purified single isomers of methyl linolenate hydroperoxide (9-, 12-, 13- and 16-hydroperoxides), a purified 9,16-dihydroperoxide of methyl linolenate (I) and 2 purified single isomers of methyl linolenate hydroperoxy cyclic peroxide [13,15-epidoxy-16-hydroperoxide (II) and 10, 12-epidioxy-9-hydroperoxide (III)] that represent all of the major product types that arise during methyl linolenate autoxidation. The thermal decomposition of these compounds has been examined under the same conditions as used by Chan et al. (5) and volatile products have been identified using gas chromatography (GC) and gas chromatography-mass spectrometry (MS).

Our results on the identification and origin of the major volatiles are to a large extent in agreement with those of Frankel et al. (6,7). Although we have observed and identified fewer components, some of them, with important sensory characteristics, are additonal to those reported by Frankel. We have also shown that, whereas the thermal decomposition at 160 C of individual methyl linoleate hydroperoxides (9- and 13-isomers) leads to extensive isomerization (involving oxygen migration between C-9 and C-13) before fragmentation of the carbon skeleton (5), similar decomposition of individual methyl linolenate hydroperoxides and cyclic peroxides results in very little, if any, isomerization before fragmentation. This is shown by the characteristic degradation products that are obtained for each individual isomer.

EXPERIMENTAL METHODS

DL-α-tocopherol (α-T) and methyl linolenate were obtained from Sigma Chemical Co. Ltd (UK). The ester was purified by column chromatography on Hiflosil (Applied Science Labs. Inc., State College, PA) immediately before use. Authentic 2,3-pentanedione, 1-penten-3-one, *trans,trans*-2,4-heptadienal, hexanal and methyl octanoate were obtained from commercial sources. 4,5-Epoxyhept-2-enal was synthesized by epoxidation (3-chloroperoxybenzoic acid) of 2,4-heptadienal (8). Analytical and preparative HPLC was carried out using Partisil-5 columns as previously described (9,10).

Hydroperoxides were prepared either by enzymic or autoxidation reactions. The 9- and 13-hydroperoxides of linolenic acid were obtained from enzymic reactions using tomato and soybean lipoxygenases, respectively (11,5). The methyl ester derivatives were prepared and purified before GC analysis. Increased yields of the 9-, 12-, 13- and 16-monohydroperoxides and the 9,16-dihydroperoxide were obtained from autoxidation of methyl linolenate carried out in the presence of α-tocopherol (5% w/w or greater) (12). Oxidations were carried out in the dark at 40 C until the conjugated diene value (9) indicated that the sample was oxidized 15-30%. The monohydroperoxide (HPO) and dihydroperoxide (DHPO) fractions from this autoxidation mixture were isolated and separated by column chromatography on Hiflosil. Column cuts were monitored by thin layer chromatography (TLC). Those containing monohydroperoxides were bulked and concentrated to a small volume. Isolation of individual HPOs was achieved by repeated injections of this concentrate onto the preparative HPLC column using conditions as previously described (10). Purification of the DHPO fraction, which from this oxidation contained only isomeric 9,16-dihydroperoxides (12), was achieved by a similar preparative HPLC procedure using 3% ethanol in hexane as eluent. Two isomeric hydroperoxy cyclic peroxides, 15,16-erythro-16-hydroperoxy-13,15-epidioxy-9-*cis*,11-*trans*-octadecadienoate (II) and 9,10-erythro-9-hydroperoxy-10,12-epidioxy-13-*trans*,15-*cis*-octadecadienoate (III), which are the major 'diperoxide' isomers obtained from autoxidation of methyl linolenate at 40 C, were separated from an autoxidation mixture using procedures previously described (10).

Individual hydroperoxide compounds (150-200 μg/injection) were thermally decomposed at the injection port of the GC and the breakdown products analyzed on a Carbowax 20M column as described by Chan et al. (5). The identities of decatrienals were confirmed by analysis on a PEGA column (13). Samples were injected with and without added methyl ester markers so that fatty acid methyl ester (FAME) numbers could be calculated (13) for each major peak detected by either flame ionization (FID) or electron capture (ECD). The exhaust of the ECD was monitored by sniffing to detect odorous volatiles. Each sample was analyzed again on a GC coupled to the MS. Identification of each volatile component was achieved by comparing its properties (GC retention, MS and odor) with authentic commercial or synthesized compounds or with data obtained in previous work (13).

RESULTS AND DISCUSSION

We have previously shown that autoxidation of methyl linolenate in the presence of 5% w/w α-tocopherol provides a means of producing reasonable yields of each of 4 isomeric hydroperoxides (9-, 12-, 13- and 16-positional isomers) having only the *cis,trans* conjugated diene configuration (9). Each of these isomers is produced in almost equal quantity and they may be separated by careful preparative HPLC where their elution order is 13-, 16-, 12- and 9-. Individual monohydroperoxides obtained in this way were reduced ($NaBH_4$) and analyzed by HPLC as the hydroxy derivatives to more accurately determine their isomeric purity. The compositions of the hydroperoxide fractions obtained are listed in Table 1. A more polar fraction from the autoxidation of methyl linolenate in the presence of α-tocopherol was found to be a mixture of isomeric conjugated triene dihydroperoxides (12). The mixture contained 4 isomers, but since they were all 9,16-dihydroperoxides (12), the purified mixture separated by preparative HPLC was used for this investigation. Reduction of this dihydroperoxide fraction ($NaBH_4$) and GC-MS analysis of the hydroxy derivatives as the *bis*-trimethylsilyl ethers confirmed that oxygenation was solely at the 9- and 16-positions.

The 2 monocylic peroxides used in this study were chosen to represent each of the 2 positional isomers that are formed by linolenate autoxidation. 9-Hydroperoxy-10,12-epidioxides are formed via the 12-hydroperoxy radical intermediate, whereas the 16-hydroperoxy-13, 15-epidoxides are formed via the 13-hydroperoxy radical intermediate. The 2 major cyclic peroxides formed as linolenate autoxidation products are the erythro-*cis*-epidioxy-*cis*, *trans*-diene isomers of these 2 structures as suggested by Mihelich (14). Note that our original assignments for this group of compounds (10) were incorrect and that in all cases threo assignments should be changed to erythro and vice versa. An independent confirmation of these structures has since been obtained (15). These 2 major products are also the first 2 peaks of the monocyclic peroxide fraction to elute during an HPLC separation and they were obtained preparatively in this way (10).

TABLE 1

Isomeric Purity of Monohydroperoxide Fractions

Monhydroperoxide fraction[a]	Actual isomeric composition (mol percent) (all positional isomers *cis,trans*)			
	13-	16-	12-	9-
13-Hydroperoxide	100	0	0	0
'16-Hydroperoxide'	4	96	0	0
'12-Hydroperoxide'[b]	5	18	77	0
9-Hydroperoxide	0	0	0	100

[a]Fractions from preparative HPLC were converted (with $NaBH_4$) to hydroxy derivatives and then analyzed for isomer composition.

[b]Hydroperoxide chromatographed twice by preparative HPLC.

A list of the volatile products detected after thermally decomposing each of the various hydroperoxides, together with their FAME numbers and odor descriptions, is given in Table 2. For the acyclic monohydroperoxides, the major volatiles were as would have been predicted for each particular isomer. Conjugated 2,4-dienals in both *cis,trans* and *trans,trans*-conjugated diene configurations were major products except for the 16-hydroperoxide, where the expected methyl 16-oxohexadeca-9,12,14-trienoate, although probably formed, was not detected because of its excessively long retention time under our GC conditions. The unsaturated long-chain (greater than C_6) aldehydes, 2,4-heptadienals from the 12-hydroperoxide and 2,4,7-decatrienals from the 9-hydroperoxide, were both given fatty odor descriptions as they eluted from the GC. The ECD response to compounds having the 2,4-dienal structure and to 4,5-epoxyhept-2-enal was very high and proved useful in their identification. The predominant characteristic volatiles were 2,4,7-decatrienals from the 9-hydroperoxide, 2,4-heptadienals from the 12-hydroperoxide and methyl 13-oxotrideca-9, 11-dienoates from the 13-hydroperoxide. The 16-hydroperoxide produced no detectable characteristic volatiles but did produce trace amounts of 2,4-heptadienals and 4,5-epoxyhept-2-enal. The formation of heptadienals from the 16-hydroperoxide might be accounted for by isomerization or could possibly have been caused by contamination of the original '16-hydroperoxide' by some 12-hydroperoxide (Table 1). The second possibility seemed unlikely as the isomeric analysis of the '16-hydroperoxide' revealed that, although it was only 96% 16-hydroperoxide, the remaining 4% was 13-hydroperoxide, and no 12-hydroperoxide was detected. A small amount of isomerization of 16-hydroperoxide to 12-hydroperoxide may have occurred. That the 9- and 13- and 12- and 16-isomer pairs would have equilibrated to some extent before decomposition by a reversible carbon-oxygen β-scission mechanism (10, 16,17), as do the 9- and 13-hydroperoxides of methyl linoleate (5), might have been pre-

TABLE 2

Analysis of Volatiles from Thermally Decomposed Hydroperoxides

Volatile	Fame number 20M	Precursor compound 9-HPO	12-HPO	13-HPO	16-HPO	(I)	(II)	(III)	Odor
1-Penten-3-one	4.5			M					Metallic, carbide
2,3-Pentanedione	4.7						S[a]		Buttery
Hexanal	5.0			M					Green
cis-3-Hexenal	5.6			M					Green, grassy
Methyl octanoate	8.0	L	M	tr	tr	L	tr	S	–
2,4-Heptadienal (*t,c*)	8.7		XL[a]		tr[a]			L[a]	Fatty, nutty
2,4-Heptadienal (*t,t*)	9.0		L[a]		tr[a]			L[a]	Fatty, nutty
4,5-Epoxyhept-2-enal	10.9		M[a]		S[a]				–
2,4,7-Decatrienal (*t,c,c*)	12.1	L[a]							Fatty, fried
2,4,7-Decatrienal (*t,t,c*)	12.7	L[a]							Fatty, fried
Methyl 9-oxononanoate	14.3	L	L	M	M	XL	S	XL	–
Methyl 13-oxotrideca-9,11-dienoates	20.9			XL[a]			L[a]		–
	21.6			XL[a]			M[a]		–
Unknowns	6.7							L	–
	7.2			S					–
	8.5		M		M			S	–
	18.3						L		–

Letters refer to FID peak heights at X 200 attenuation $\equiv 2 \times 10^{-10}$ amp; tr = 5% or less; S = 6-10%; M = 11-20%; L = 21-80%; XL = >80% of full scale deflection (fsd).

[a]Indicates a large ECD response 60% fsd or more (detector saturation) at attenuation $5 \times 10^{3} \equiv 5 \times 10^{-9}$ amp.

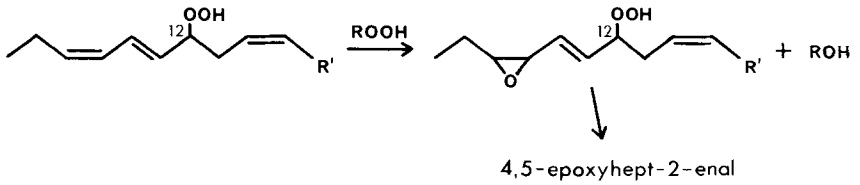


FIG. 1. Proposed pathway to 4,5-epoxyhept-2-enal from methyl 12-hydroperoxy-9,13, 15-octadecatrienoate.

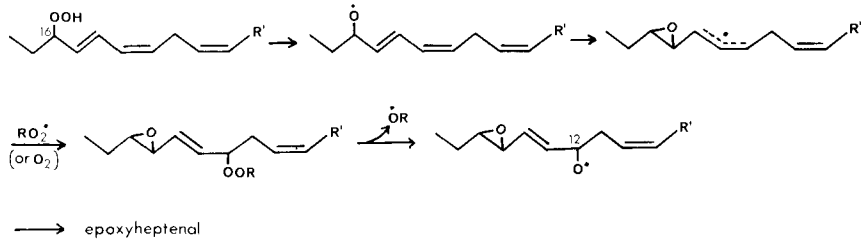


FIG. 2. Proposed pathway to 4,5-epoxyhept-2-enal from methyl 16-hydroperoxy-9,12,14-octadecatrienoate.

dicted. The evidence for this type of isomerization occurring with linolenate hydroperoxides under GC conditions is, on the whole, very weak. For example, no trace of the methyl 13-oxotrideca-9,11-dienoates, which are the major volatiles produced from the 13-hydroperoxide, was obtained from the 9-hydroperoxide and, conversely, no trace of 2,4,7-decatrienals, which are the major volatiles produced from the 9-hydroperoxide, was obtained from the 13-hydroperoxide. However, methyl 9-oxononanoate, which would only be predicted to arise directly from the 9-hydroperoxide, was also produced, not only from the 13-hydroperoxide but also from the 12- and 16-isomers. An entirely satisfactory mechanism for this latter observation cannot be provided.

Frankel et al. (6) have presented evidence for the isomerization of oleate hydroperoxides (9-,10-OOH → 8-,9-,10-,11-OOH) before thermal decomposition at 210 C. They failed to show significant isomerization of linoleate hydroperoxides (6) but this was because they only examined mixtures already containing equal proportions of 9- and 13-, and 10- and 12-isomers. In agreement with our results, they also did not observe any significant isomerization of linolenate hydroperoxides (6), although a small amount of isomerization would have been difficult to detect with the mixtures of isomers that they studied.

The formation of 4,5-epoxyhept-2-enal from both the 12- and 16-hydroperoxides requires some comment. From the 12-hydroperoxide, a simple epoxidation mechanism (Fig. 1), using hydroperoxide as the oxidizing agent, could account for the formation of the immediate precursor, epoxyhydroperoxide.

The formation of 4,5-epoxyhept-2-enal from the 16-hydroperoxide could proceed via the mechanism shown in Figure 2.

Alternatively, the epoxyhydroperoxide could have existed as a trace component in the original hydroperoxide sample. 4,5-Epoxyhept-2-enal was first identified in the volatiles collected from an oxidized butterfat system (8) but has since been identified in the breakdown products of a thermally decomposed mixture of methyl linolenate hydroperoxides (6).

The volatiles, 1-penten-3-one, *cis*-3-hexenal and hexanal, were only observed from the decomposition of the 13-hydroperoxide. Formation of 1-penten-3-one can be explained by a mechanism (Fig. 3) involving oxidation of the allylic radical derived from the 13-hydroperoxide.

Cis-3-hexenal is the expected product from the 13-hydroperoxide but the formation of hexanal is not so easily explained. The obvious precursor for hexanal formation would be the 13-hydroperoxide of linoleate. Trace amounts of methyl linoleate in the methyl linolenate used in this study could be responsible for this product as the respective 13-hydroperoxides are very similar chromatographically.

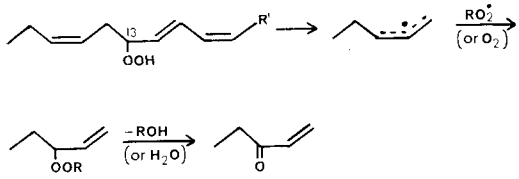
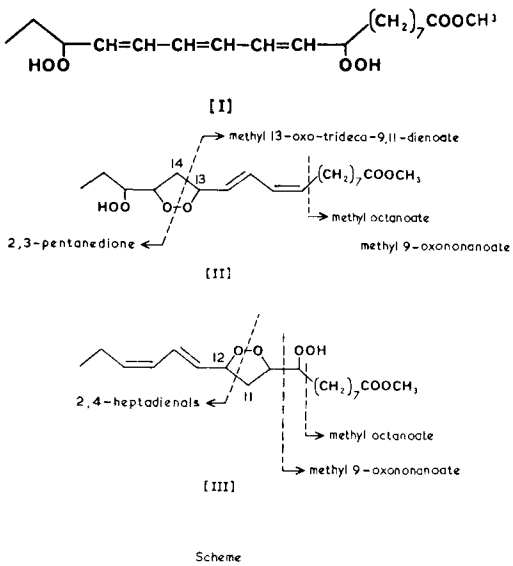


FIG. 3. Proposed pathway to 1-penten-3-one from methyl 13-hydroperoxy-9,11,15-octadecatrienoate.

Only 2 volatile products were observed from thermal decomposition of the dihydroperoxide (I) (Table 2). These were methyl octanoate and methyl 9-oxononanoate, both arising from the carboxyl end of the molecule. Although ethane and propanal would have been expected products, these were not detected as they would not have been separated from the solvent peak (hexane) of the gas chromatogram. No conjugated aldehyde or dialdehyde products, which might also have been expected, were detected, but they may have been too unstable to survive the thermal treatment.

Thermal decomposition of both hydroperoxy cyclic peroxides (II) and (III) gave rise to 2,4-dienal products. The cyclic peroxide (III) gave only 4 identifiable products. Methyl 9-oxononanoate was the major product, which, together with methyl octanoate, was an expected product. *Cis,trans* and *trans,trans*-2,4-heptadienals were the other 2 products, whose formation would require an oxygen-oxygen cleavage of the cyclic peroxide ring and a carbon-carbon cleavage between carbons 11 and 12 of the chain (Scheme).



Scheme

Frankel et al. (7) identified these components plus many others from the thermal decomposition of the peroxide (III) but he used a higher decomposition temperature (200 C), which would be likely to cause more extensive decomposition, and he also used capillary GC, which would be more sensitive for the detection of additional minor components. No evidence for the formation of heptenal or heptanol, which were significant products (≥2,4-heptadienals) under Frankel's conditions, was obtained in our work. The major decomposition products from the peroxide (II) were the isomeric methyl 13-oxo-trideca-9,11-dienoates, although these had no odor. Methyl octanoate and methyl 9-oxononanoate were minor products and, in this case, the formation of the 9-oxononanoate is less readily explained. Frankel et al. (7) suggested that formaldehyde was involved in its formation. Although this is possible, an alternative mechanism (Fig. 4) involving peroxy radical isomerization via a β-scission mechanism could account for both methyl octanoate and methyl 9-oxononanoate.

However, this mechanism would equally be expected to lead to the formation of decatrienals, which were not observed as decomposition products of the hydroperoxy cyclic peroxide (II). No evidence for the formation of methyl furan octanoate, which was a major product (5.7%) under Frankel's conditions, was obtained in our work. The most interesting product from the thermal decomposition of II was 2,3-pentanedione, which has a strong ECD response and a strong butterlike smell. This product was not observed by Frankel et al. (7). Although present in only small amounts, its presence was confirmed by all our identification methods. Its formation would require an oxygen-oxygen cleavage of the cyclic peroxide ring followed by a carbon-carbon cleavage between carbons 13 and 14 and, finally, a loss of water (Scheme).

Some of the volatile decomposition products reported here (1-penten-3-one, 2, 3-pentanedione and *cis*-3-hexenal) have been associated with the "reversion flavor" that develops when refined soybean oil in stored. 2,3-Pentanedione has also been blamed for the buttery flavor of oxidized soybean oil (18) and a cyclic peroxide of linolenic acid has been suggested as its precursor. The evidence presented here supports that suggestion.

ACKNOWLEDGMENTS

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REFERENCES

1. Frankel, E.N. (1983) Prog. Lipid Res. 22, 1-33.
2. Haslbeck, F., and Grosch, W. (1983) Lipids 18, 706-713.
3. Bascetta, E., Gunstone, F.D., and Walton, J.C. (1983) J. Chem. Soc. Perkin Trans. II, 603-613.
4. Evans, C.D., List, G.R., Dolev, A., McConnell, D.G., and Hoffmann, R.L. (1967) Lipids 2, 432-434.
5. Chan, H.W.-S., Prescott, F.A.A., and Swoboda, P.A.T. (1976) J. Am. Oil Chem. Soc. 53, 572-576.
6. Frankel, E.N., Neff, W.E., and Selke, E. (1981) Lipids 16, 279-285.

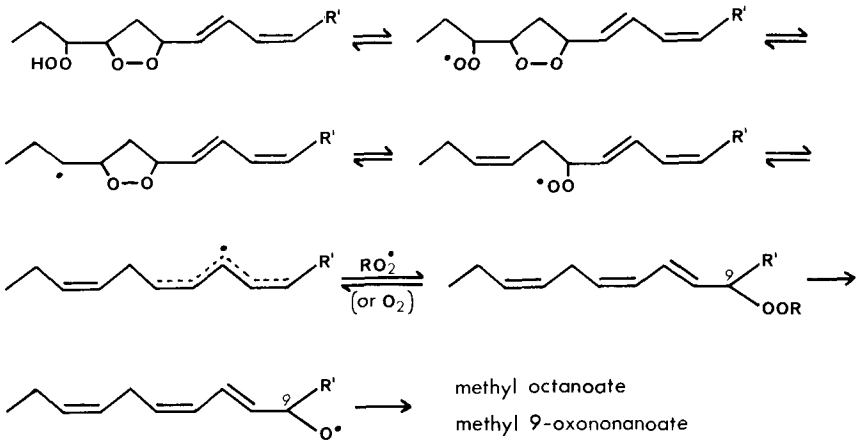

FIG. 4. Tentative pathway to methyl octanoate and methyl 9-oxononanoate from methyl 16-hydroperoxy-13,15-epidioxy-9,11-dienoate.

7. Frankel, E.N., Neff, W.E., and Selke, E. (1983) Lipids 18, 353-357.
8. Swoboda, P.A.T., and Peers, K.E. (1978) J. Sci. Fd. Agric. 29, 803-807.
9. Peers, K.E., Coxon, D.T., and Chan, H.W.-S. (1981) J. Sci. Fd. Agric. 32, 898-904.
10. Coxon, D.T., Price, K.R., and Chan, H.W.-S. (1981) Chem. Phys. Lipids 28, 365-378.
11. Matthew, J.A., Chan, H.W.-S., and Galliard, T. (1977) Lipids 12, 324-326.
12. Coxon, D.T., Peers, K.E., and Rigby, N.M. (1984) J. Chem. Soc. Chem. Commun., 67-68.
13. Swoboda, P.A.T., and Peers, K.E. (1977) J. Sci. Fd. Agric. 28, 1010-1018.
14. Mihelich, E.D. (1980) J. Am. Chem. Soc. 102, 7143, footnote 19.
15. Coxon, D.T. and Price, K.R. (1984) J. Chromatogr., 285, 392-394.
16. Chan, H.W.-S., Levett, G., and Matthew, J.A. (1979) Chem. Phys. Lipids. 24, 245-256.
17. Porter, N.A., Lehman, L.S., Weber, B.A., and Smith, K.J. (1981) J. Am. Chem. Soc. 103, 6447-6455.
18. Seals, R.G., and Hammond, E.G. (1970) J. Am. Oil Chem. Soc. 47, 278-280.

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Regulation of Phosphatidylinositol Turnover, Calcium Metabolism and Enzyme Secretion by Phorbol Dibutyrate in Neutrophils

CAROLINE M. KRAMER,[a,1] **RICHARD C. FRANSON,**[b] and **RONALD P. RUBIN,**[a]
Departments of Pharmacology[a] *& Biochemistry,*[b] *Medical College of Virginia, Richmond, VA 23298*

ABSTRACT

The action of the tumor promoter, phorbol 12,13-dibutyrate (PDBu), on rabbit peritoneal and human neutrophils is associated with stimulation of ^{14}C-arachidonic acid incorporation into phospholipids within 1-2 min. Stimulated ^{14}C-arachidonate incorporation was relatively selective for phosphatidylinositol (PI) in rabbit neutrophils. In contrast, the secretory response of human neutrophils to PDBu coincided with stimulated label incorporation into phosphatidylserine (PS), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidic acid (PA) and PI. Significant increases in label incorporation were observed with PDBu concentrations as low as 2 nM, and the dose response of stimulated label incorporation paralleled that of evoked lysozyme secretion. A parallel, but partial, inhibition of PDBu-stimulated PI labeling and enzyme release was observed after exposing rabbit neutrophils to calcium-deprived medium, whereas calcium deprivation failed to significantly depress either of these stimulant actions of PDBu in human neutrophils. Further, in rabbit neutrophils PDBu elicited an increase in cell associated ^{45}Ca. However, PDBu was unable to promote the incorporation of ^{32}P orthophosphate into PI or enhance phospholipase A_2 activity in broken cells. These findings suggest that one expression of the interaction between phorbol esters and their receptors on neutrophils involves the turnover of arachidonic acid in phospholipids. This stimulated turnover of arachidonate may be a critical step in the cascade of events associated with neutrophil activation.
Lipids 19: 315-323, 1984.

INTRODUCTION

Activation of the neutrophil by the chemotactic peptide formyl-Met-Leu-Phe and the calcium ionophore A23187 is accompanied by calcium influx and remodeling of membrane phospholipids, particularly phosphatidylinositol (PI) (1-4). Ionophore A23187 and formyl-Met-Leu-Phe stimulate a parallel arachidonic acid turnover in PI and enzyme secretion by a calcium-dependent process (3,4). We (3,4) have hypothesized that the influx of calcium promoted by these secretagogues activates phospholipase A_2 to initiate a deacylation-reacylation cycle of arachidonic acid in PI. This cycle, catalyzed by phospholipase A_2 and acyl CoA-transferase, represents a potentially pivotal mechanism for regulating neutrophil function. Lysophospholipids formed as intermediates of this sequence have been implicated in membrane fusion phenomena, e.g., exocytotic secretion (5). Further, hydroxylated derivatives of arachidonic acid may act as second messengers to modulate secretion (6,7).

In an effort to provide further insight into the roles of calcium and arachidonate turnover in secretion, we have used the phorbol esters as stimuli of neutrophils. These tumor promoters trigger the release of the contents of secondary granules (8,9) by a mechanism presumed to involve the mobilization of cellular calcium (10,11). Moreover, phorbol esters have been reported to stimulate phospholipase A_2 and arachidonate metabolism in several cell types (12-14), including the neutrophil (15). We have employed phorbol 12,13 dibutyrate (PDBu), a less lipophilic but highly active phorbol ester that binds to the same cellular receptors as 12-0-tetradecanoyl phorbol-13-acetate (TPA) (16). This report reveals that the PDBu-evoked secretory response of the neutrophil is not accompanied by the activation of phospholipase C but is associated with an alteration in calcium handling and a stimulation of arachidonic acid incorporation into PI and other phospholipids. The implications of these findings with regard to neutrophil activation are discussed.

MATERIALS AND METHODS

Materials

^{14}C-Arachidonic acid (58.4 mCi/mmol) was obtained from Amersham/Searle Corporation, Arlington Heights, IL. Other radioactive

[1] Dr. Kramer's present address is: Department of Pharmacology; University of Virginia School of Medicine; Charlottesville, VA 22908

materials were obtained from New England Nuclear Corp., Boston, MA. Cytochalasin B, phosphocreatine, creatine phosphokinase, adenosine triphosphate, fatty acid free bovine serum albumin, Hepes, p-nitrophenyl-N-acetyl-glucosaminide, TPA, PDBu, and *Micrococcus lysodeikticus* were obtained from Sigma Chemical Company, St. Louis, MO. Precoated thin layer chromatographic (TLC) plates (Silica Gel G) were obtained from Merck Darmstadt, Cincinnati, OH. All chemicals were reagent grade. ^{14}C-Arachidonic acid was stored in toluene under argon at −40 C. The desired amount of ^{14}C-arachidonate was evaporated to dryness under nitrogen, redissolved in a small volume of absolute ethanol and suspended in an incubation medium. The final concentration of ethanol, 0.1% (v/v) in the incubation medium, did not alter secretion or label incorporation. PDBu and cytochalasin B were stored in DMSO at concentrations of 1 mg/ml at −70 C and diluted in incubation medium to appropriate concentrations before use. The final concentration of DMSO, 0.1% (v/v) or less, did not alter secretion or incorporation of radiolabel.

Cell Isolation

Rabbit peritoneal neutrophils were prepared according to the method described previously (3) with the following modifications: albino rabbits received intraperitoneal injections of 150 ml 0.1% glycogen in sterile 0.9% NaCl, and the peritoneal exudate was collected in heparinized flasks 6-12 hr later. Contaminating erythrocytes were removed by hypotonic lysis. Cells were washed and resuspended in a modified Earle's balanced salt solution containing 0.5 mM Ca^{2+}, 0.2 mM Mg^{2+}, 20 mM Hepes and 0.025% bovine serum albumin.

Human neutrophils were obtained by the method of Boyum (17). Heparinized whole blood was obtained from healthy human volunteers, and neutrophils were separated by the following procedure. A 40 ml aliquot of heparinized blood was mixed with 10 ml 6.0% Dextran T-500 (Pharmacia, Piscataway, NJ) and allowed to settle for 30 min at 37 C. Plasma was removed with a sterile pipette and mixed with an equal volume of sterile saline. A 25 ml aliquot of plasma/saline was layered over 12.5 ml Ficoll-Paque (Pharmacia, Piscataway, NJ) and centrifuged at 600 × *g* for 30 min at 37 C. A pellet of neutrophils was obtained, and the supernatant was discarded. Erythrocytes were removed by hypotonic lysis for 30 sec using 20 ml water, and lysis was stopped with 10 ml 2.7% NaCl. With this procedure, a preparation of greater than 95% purity was obtained, contaminated only by some additional granulocytes. Cells were resuspended in modified Earle's balanced salt solution, counted and the cell concentration adjusted to 16×10^6/ml.

All cells were preincubated at 37 C with cytochalasin B (1 μg/ml) for 5 min before the start of each experiment to depress motile and phagocytic responses (2,3). For calcium deprivation experiments, cells were resuspended in buffer lacking added $CaCl_2$ and containing 0.4 mM EGTA. No release of the cytoplasmic enzyme LDH was observed, indicating that cell viability was maintained.

Incorporation of Radiolabeled Precursor into Cellular Phospholipid

Radiolabel incorporation experiments were initiated by adding 4×10^6 cells in 250 μl to an equal volume of buffer containing 0.03 μCi 1-^{14}-arachidonic acid, with or without stimulant, in a shaking water bath at 37 C under an atmosphere of 95% O_2 and 5% CO_2. For ^{32}P-phospholipid turnover studies, 4×10^6 cells in 250 μl, which were preincubated in 50 μCi/ml ^{32}P-orthophosphate for 30 min, were added to an equal volume of buffer with or without stimulant. Reactions were terminated by adding 3 ml $CHCl_3$/MeOH (1:2) and lipids were extracted for at least 2 hr. Phospholipids were extracted by the method of Bligh and Dyer (18) and separated by thin layer chromatography (TLC), employing 2 solvent systems run in a single dimension (19). Phospholipids were visualized by iodine and identified by comparing to standards, scraped into vials and counted for 10 min in 5 ml Beckman EP using a liquid scintillation spectrometer. Background counts—averaging 14 cpm—were subtracted from all samples.

Enzyme Assays

Enzyme release experiments were identical to those described above except that radiolabeled compounds were omitted and reactions were terminated by rapid centrifugation at room temperature. The supernatant was removed and cell pellet lysed with 0.1% Triton X-100 for 10 min at room temperature. Soluble cell contents were obtained from the supernatant after centrifugation of this lysate. Lysozyme was measured by the rate of lysis of a *Micrococcus lysodeikticus* solution, as reflected in a decrease in absorbance at 450 nm (20). Lactate dehydrogenase and N-acetyl-glucosaminidase activities were measured as described previously (21,22). Enzyme release was expressed as the percentage of total cell enzyme activity, obtained by dividing activity in the supernatant by total cell (supernatant

plus lysed cells) activity. Basal secretion was substracted from corresponding values for stimulated secretion.

Phospholipase A_2 activity was determined by suspending rabbit neutrophils in the following buffer, which provides an ATP regenerating system: NaCl (20 mM); KCl (100 mM); $MgCl_2$ (5 mM); $NaHPO_4$ (0.96 mM); $NaHCO_3$ (25 mM); creatine phosphate (5 mM); creatine phosphokinase (5 units/ml); EGTA (1 mM); and $CaCl_2$ (final concentration 0.2 μM). Cells were adjusted to a concentration of 16×10^6/ml in this buffer, disrupted by sonication for 2 min and assayed for phospholipase A_2 activity (23). Aliquots (25 μl) of sonicated cells were added to tubes containing autoclaved 1-^{14}C-oleate labeled *E. coli*, buffer and PDBu and ATP to a final volume of 500 μl. *E. coli* phospholipids were labeled almost exclusively in position 2. Ca. 2.5×10^8 cells containing 6,000 cpm in 5 nmol of phospholipid were used in each assay. The final concentration of ATP was 1.5 mM; high calcium samples contained buffer with 2 μM $CaCl_2$. Samples were incubated for 5 min at 37 C and stopped with $CHCl_3$/MeOH (1:2). Lipids were extracted (18), organic phases evaporated to dryness under nitrogen and resuspended in 80 μl $CHCl_3$/MeOH (9:1). Lipids were applied to heat-activated Silica Gel G-25 thin layer plates (Brinkmann) that were developed in petroleum ether/ethyl ether/acetic acid (80:20:1, v/v/v). Phospholipid, which remained at the origin, and fatty acids corresponding to standards, were visualized by iodine, scraped into minivials containing 5 ml Beckman EP and radioactivity was counted. Phospholipase A_2 activity, as monitored by fatty acid release from position 2 labeled phospholipids, was determined by dividing radiolabel in fatty acid by label in fatty acids plus phospholipid of each sample. Nonenzymatic hydrolysis, which was always less than 1%, was subtracted from each value.

Net ^{45}Ca Flux

Rabbit neutrophils were initially incubated with 4 μCi/ml ^{45}Ca for 40 min to allow sufficient time for the radioactivity to reach steady state levels before the addition of 10 nM formyl-Met-Leu-Phe or 2 μM PDBu. Net ^{45}Ca uptake of samples of cell suspension was determined (24), and ^{45}Ca contents expressed as nanomoles per mg of cell protein.

RESULTS

Arachidonyl-PI Turnover and Secretion in Rabbit Neutrophils

Time course. Rabbit neutrophils accumulated arachidonic acid in PI during a 30 min incubation (Table 1). More than 75% of the radioactivity associated with PI after 30 min incubation with ^{14}C-arachidonate was released by treatment of purified PI with exogenous phospholipase A_2 and recovered in the zone corresponding to free arachidonic acid. Thus, most of the radiolabel was attributed to unsaturated fatty acid in position 2 of this phospholipid.

PDBu (20 nM) elicited a prompt stimulation of arachidonate incorporation into PI (Table 1). When values from stimulated cells were represented as a percentage of values derived from control cells at a corresponding time point, a 12% stimulation of labeling was detectable within 2 min (Table 1). Maximal levels of stimulation were approached by 5 min and were maintained for at least 30 min. Similarly, the maximal rate of enzyme secretion was observed within the first 5 min and after 15 min secretion was essentially complete (Fig. 1). No other phospholipid was similarly affected by PDBu during the time course of the experiment, except that a significant increase in labeling of phosphatidylserine ($16 \pm 2\%$) was observed after 30 min.

Dose-response relationship. An increase in labeling of PI was detectable with 2 nM PDBu and label incorporation continued to rise with increasing concentrations as high as 2 μM PDBu (Fig. 2). Labeling of other phospholipids was not dose related, indicating that PDBu-stimulated arachidonic acid incorporation was selective for PI. Both PI labeling and lysozyme release were stimulated in parallel in the concentration range of 2 nM to 2 μM PDBu (Fig. 2).

TABLE 1

Time-Course of ^{14}C-Arachidonic Acid Incorporation into Phosphatidylinositol and the Effect of PDBu

Time (min)	^{14}C-Arachidonic acid incorporation cpm/4 X 10^6 cells		Percentage of control
	Control	PDBu	
2	260 ± 30	290 ± 30	112 ± 2 *
5	790 ± 150	960 ± 270	122 ± 2 *
15	2280 ± 340	2750 ± 140	125 ± 5 *
30	2660 ± 670	3380 ± 240	127 ± 6 *

Rabbit neutrophils (4 X 10^6 in 250 μl) preincubated for 5 min in the presence of cytochalasin B (1 μg/ml) were added to an equal volume of buffer containing 0.03 μCi 1-^{14}C-arachidonic acid with or without 20 nM PDBu. The experiment was stopped at various intervals by adding 3 ml $CHCl_3$/MeOH (1:2) and radiolabeled PI was determined as described in the Materials and Methods section. Values are means (± SE) for 4-9 independent experiments, each done in duplicate.

*$P < 0.05$ vs control, unpaired *t*-test.

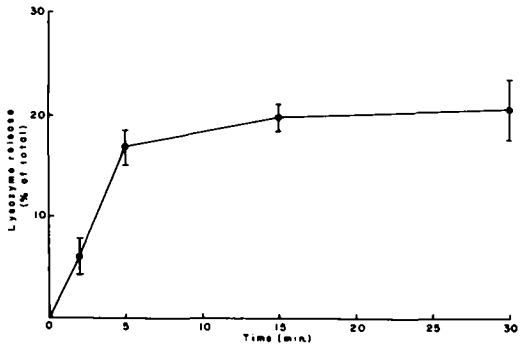

FIG. 1. Temporal profile of lysozyme release from neutrophils stimulated with PDBu. Rabbit neutrophils preincubated with cytochalasin B were incubated in the absence or presence of 20 nM PDBu for various times and lysozyme release was determined as described in Materials and Methods. Values shown are means (± SE) of at least 4 determinations. According to 2-way analysis of variance, lysozyme release was significantly enhanced by PDBu over time ($P < 0.05$).

The continued rise in label incorporation with 2 μM PDBu coincided with the onset of N-acetyl-glucosaminidase secretion (4.1 ± 0.8%) (n = 5), although no detectable release of cytoplasmic lactate dehydrogenase was found (results not shown).

Effect of calcium deprivation. A previous study demonstrated a parallel and profound reduction in formyl-Met-Leu-Phe evoked enzyme release and (arachidonyl)-PI turnover when neutrophils were incubated in a medium devoid of calcium (3). When neutrophils were washed and incubated in a calcium-free medium, a parallel–but partial–depression of PDBu-induced lysozyme secretion and ^{14}C-arachidonate incorporation into PI was observed (Fig. 3).

Effect of PDBu on ^{32}P-Orthophosphate Incorporation into Phospholipids

A high concentration of PDBu (2 μM) failed to increase significantly the labeling of phosphatidic acid (PA) or PI during a 10 min incubation period (Fig. 4). By contrast, as previously reported (2,3), formyl-Met-Leu-Phe

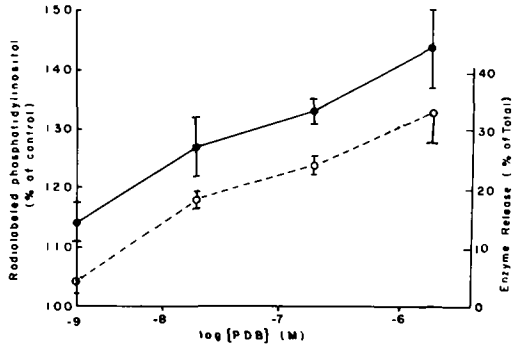

FIG. 2. PDBu dose-response curves of PI labeling and enzyme release. Rabbit neutrophils preincubated with cytochalasin B were incubated in the presence of increasing concentrations of PDBu with 0.03 μCi 1-^{14}C-arachidonic acid for 30 min and labeling of PI measured (solid line). Incorporation is expressed as a percentage of basal incorporation from paired samples. All values are means (± SE) of 3-8 determinations. Radiolabel incorporation was significantly enhanced compared with control at all concentrations of PDBu, as determined by unpaired t-test ($P < 0.05$). Lysozyme release was determined in neutrophils that were incubated with increasing concentrations of PDBu for 15 min (dotted line). Values shown are means (± SE) of at least 4 determinations. According to 2-way analysis of variance, lysozyme release was significantly enhanced compared with control with increasing concentrations of PBDu ($P < 0.05$).

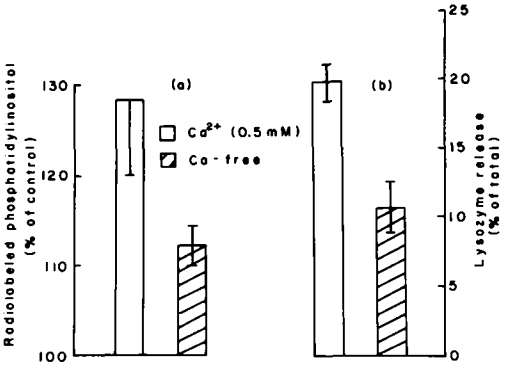

FIG. 3. Effect of calcium deprivation on PDBu-stimulated ^{14}C-arachidonic incorporation into PI and lysozyme release. (a) Rabbit neutrophils preincubated with cytochalasin were incubated for 30 min with 1-^{14}C-arachidonic acid in either normal or calcium-deprived medium in the presence or absence of 20 nM PDBu. Phospholipids were extracted and processed as described in Materials and Methods. Bars represent means (± SE) of at least 6 determinations. Incorporation of label into PI was significantly enhanced by PDBu in the presence or absence of calcium, as determined by unpaired t-test. (b) Bars represent mean values (± SE) for lysozyme release derived from at least 7 determinations. Lysozyme release was significantly stimulated after 15 min in the presence and absence of calcium according to unpaired t-test. Two-way analysis of variance showed that calcium enhanced the stimulatory effect of PDBu on lysozyme release ($P < 0.05$).

promoted ^{32}P incorporation into PA within 1 min (Fig. 4), followed by a rise in label incorporation into PI by 2 min (Fig. 4).

Effect of PDBu on ^{45}Ca Content of Rabbit Neutrophils

Figure 5 illustrates the comparative effects of stimulation by PDBu and formyl-Met-Leu-Phe on ^{45}Ca content of neutrophils. A maximal stimulating concentration of PDBu elicited a slowly developing increase in the ^{45}Ca content of cytochalasin-treated neutrophils under steady-state conditions. The PDBu-induced increase in cell-associated ^{45}Ca was significant after 2 min, at which time both PDBu stimulated incorporation of arachidonic acid into PI and enzyme release became significant (Table 1, Fig. 1). The more rapid and powerful stimulating action of formyl-Met-Leu-Phe on ^{45}Ca accumulation (Fig. 5) is consistent with our previous findings of a more rapid and potent action of this peptide on arachidonate turnover and enzyme secretion (3).

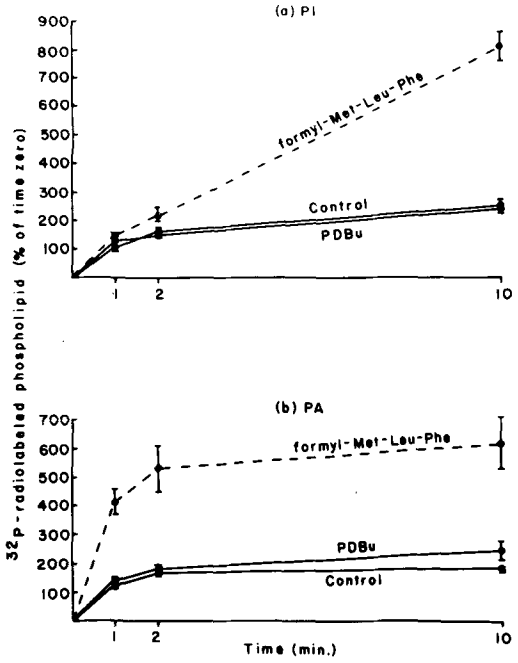

FIG. 4. Effect of PDBu and formyl-Met-Leu-Phe on ^{32}P-orthophosphate incorporation into PI and PA. Rabbit neutrophils preincubated for 30 min with cytochalasin B and ^{32}P-orthophosphate (50 μCi/ml) were incubated at time zero with radiolabel in the presence or absence of 2 μM PDBu or 10 nM F-Met-Leu-Phe. Labeling of (a) PI and (b) PA was measured at different times. Incorporation is expressed as a percentage of incorporation at time zero. All values are means (± SE) of 5 determinations. PDBu had no effect on ^{32}P incorporation at any time point according to paired *t*-test analysis. Formyl-Met-Leu-Phe significantly enhanced incorporation of ^{32}P into PA and PI after 1 and 2 min, respectively (paired *t*-test) ($P < 0.05$).

Effect of PDBu on Phospholipase A_2 Activity in Rabbit Neutrophils

The effect of PDBu on phospholipase A_2 activity in cell fragments was investigated to determine whether phorbol esters are capable of directly stimulating this enzyme (Table 2). In contrast to marked stimulation elicited by calcium, a broad range of PDBu concentrations failed to enhance enzyme activity.

PDBu-Stimulated Arachidonic Acid Turnover and Secretion in Human Neutrophils

For comparative purposes, additional experiments were conducted using human neutrophils. The relative distribution of 1-^{14}C-arachidonic acid in human neutrophil phospholipids is given in Table 3. Initially, the radiolabel was incorporated preferentially into PI and PA. Although these 2 phospholipids represent only ca. 6% of the total endogenous phospholipids in human neutrophils (25), more than 50% of the radiolabel in all phospholipids was incorporated into

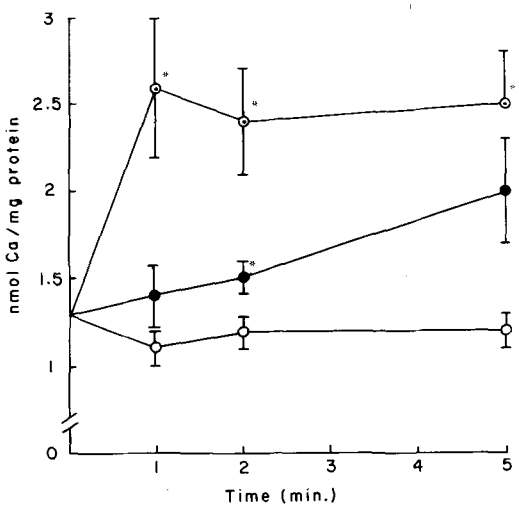

FIG. 5. Effect of PDBu and formyl-Met-Leu-Phe on ^{45}Ca content of neutrophils. Rabbit neutrophils were preincubated with ^{45}Ca for 40 min before being challenged with 2 M PDBu (•) or 10 nM formyl-Met-Leu-Phe (⊙). The values shown are means (± SE) of 4-6 determinations carried out on 2-4 different preparations. (○) Control values in the absence of stimulant. *Significantly different from control values ($P < 0.05$).

PI and PA of unstimulated cells within 2 min. The relative amount of label in PA declined after 2 min, although labeling of the other phospholipids proceeded in a linear manner for 15 min (Table 3).

PDBu elicited a concentration-dependent stimulation of arachidonate incorporation into PI and lysozyme secretion (Fig. 6). Label incorporation into PI was enhanced by PDBu compared with the control over the concentration range studied, reaching a maximum level with 200 nM PDBu (Fig. 6). Radioactivity in PA was enhanced by up to 20% over the control in a concentration-dependent manner up to 20 nM PDBu; higher concentrations of PDBu failed to stimulate labeling (data not shown).

In human neutrophils, PDBu also promoted a parallel time-dependent labeling of phospholipids and secretion of lysozyme (Fig. 7). Stimulated incorporation of ^{14}C-arachidonate into PI was significantly enhanced within 1-2 min and remained elevated for at least 15 min (Fig. 7). Similarly, a maximum rate of lysozyme secretion was attained within the first min (Fig. 7). At concentrations of PDBu above 20 nM, release of the primary granule enzyme NAGA was also detected after 1 min (not shown). This result contrasts with that obtained with rabbit neutrophils in which PDBu concentrations as high as 2 μM stimulated only lysozyme release.

In human neutrophils, in contrast to rabbit neutrophils, PDBu also promoted a concentration and time-dependent incorporation of label into other phospholipids. Arachidonate incorporation into PA increased 62 ± 8% at 1 min after the addition of PDBu, then subsequently declined to control levels by 5 min. This decline

TABLE 2

Effect of PDBu and Calcium on Phospholipase A_2 Activity in Sonicates of Neutrophils

Additions	Hydrolysis (%)
None	0.18 ± 0.09
PDBu (.01 μM)	0.18 ± 0.05
PDBu (0.1 μM)	0.15 ± 0.05
PDBu (1 μM)	0.18 ± 0.05
$CaCl_2$ (2 μM)	10.75 ± 1.06

Rabbit neutrophils were sonicated for 30 sec and aliquots (25 μl) were incubated in ATP regenerating medium for 5 min. Phospholipase A_2 was assessed as described in Materials and Methods and units are expressed as percentage of hydrolysis of *E. coli* phospholipids. Values are means (± SE) of 4 experiments derived from 2 different preparations.

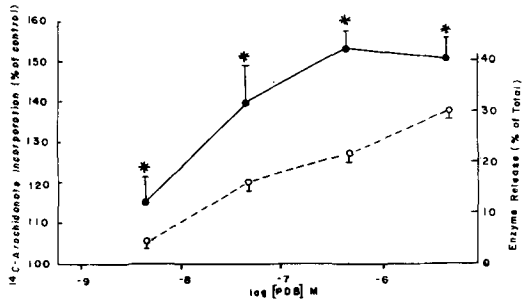

FIG. 6. Concentration dependence for PDBu stimulated ^{14}C-arachidonate incorporation into PI and lysozyme secretion. Human neutrophils were incubated for 5 min with concentrations of PDBu indicated plus 0.05 μCi 1-^{14}C-arachidonic acid. Radioactivity in PI was determined as in Materials and Methods (solid line). Values are means (± SEM) of 6-16 experiments, each performed in duplicate. *Incorporation into PI was enhanced at all PDBu concentrations ($P < 0.05$, unpaired *t*-test). Enzyme secretion was also determined in human neutrophils incubated for 5 min with the indicated concentrations of PDBu. Values for enzyme secretion (dotted line) are means (± SEM) of 3-12 determinations.

TABLE 3

Time-Course of 1-^{14}C-Arachidonic Acid Incorporation into Phospholipids of Human Neutrophils

Time (min)	cpm/4 X 10^6 cells				
	PC	PI	PS	PE	PA
1	103 ± 14	63 ± 14	13 ± 2	31 ± 3	63 ± 9
2	322 ± 33	232 ± 16	44 ± 6	78 ± 7	241 ± 18
5	499 ± 33	392 ± 23	96 ± 9	133 ± 7	203 ± 12
10	1125 ± 54	676 ± 63	207 ± 15	180 ± 13	191 ± 18
15	1313 ± 92	832 ± 69	168 ± 12	210 ± 17	178 ± 49

Cells pretreated with cytochalasin B were incubated with 0.05 μCi 1-^{14}C-arachidonic acid for various periods. Labeling of phospholipids was determined as described in Experimental Procedures. Values are means (± SEM) of 6-20 experiments.

may represent redistribution or release of label caused by the rapid turnover of PA. In addition, a 5-min period of stimulation by PDBu promoted arachidonate incorporation into phosphatidylserine (PS), phosphatidylcholine (PC) and phosphatidylethanolamine (PE) by 55 ± 7%, 55 ± 4% and 45 ± 4%, respectively. Thus, in contrast to the selectivity observed with rabbit neutrophils, PDBu-stimulated arachidonic acid incorporation into phospholipids of human neutrophils is not selective for PI. As in the rabbit neutrophil, where calcium deprivation elicited a partial impairment of PDBu induced stimulation of (arachidonyl)PI turnover and lysozyme secretion, in human neutrophils the stimulant actions of PDBu on these parameters were partially affected by calcium deprivation. In the presence of calcium, a 5 min exposure to 200 nM PDBu promoted ^{14}C-arachidonate incorporation into PI and lysozyme secretion by 54 ± 4% of basal and 22 ± 2% of the total, respectively; whereas, in the presence of 0.4 mM EGTA, arachidonate incorporation and lysozyme secretion were increased by 39 ± 7% and 17 ± 4% (n = 4-12).

DISCUSSION

Our previous investigations have provided evidence that a deacylating-reacylating mechanism that acts on the arachidonic acid moiety in position 2 of PI is correlated with the evoked release of secretory product from rabbit neutrophil by agents known to promote calcium influx (3,4). The present study has confirmed and extended these studies by demonstrating

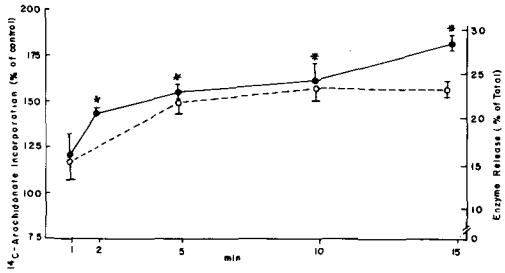

FIG. 7. Time course of PDBu stimulated ^{14}C-arachidonate incorporation into PI and enzyme release. Human neutrophils were incubated with 20 nM PDBu for the times indicated, plus radiolabeled arachidonic acid (solid line). Values are means (± SEM) of 6-12 experiments, each performed in duplicate. *Label incorporation into PI was enhanced relative to control at 2-15 min (unpaired *t*-test). Enzyme secretion was also measured in human neutrophils that were incubated with 200 nM PDBu for the times indicated. Values for enzyme secretion (dotted line) are means (± SEM) of 3-12 determinations.

that the stimulation of (arachidonyl)PI turnover is a mode of expression of the stimulant actions of PDBu, which does not promote a large influx of calcium, in both rabbit and human neutrophils. Another phorbol ester, TPA, elicited qualitatively and quantitatively similar effects on the labeling of PI and enzyme secretion in rabbit neutrophils, although the latency of responses was somewhat greater (data not shown). Although TPA elicits a modest release of arachidonic acid from prelabeled rabbit neutrophils (15), the reported inability of the TPA to stimulate release of arachidonic acid from prelabeled human neutrophils (26) may be explained by a very rapid reacylation obscuring the earlier deacylation.

The turnover of arachidonic acid in position 2 of PI is correlated with enzyme release as shown by the following findings: (a) the parallel time-course of PDBu-stimulated arachidonate incorporation and enzyme secretion; (b) the correlation between the dose-response curves of lysozyme secretion and PI-labeling over a broad range of PDBu concentrations; (c) the parallel reduction in both parameters induced by calcium deprivation in rabbit and human neutrophils. On the other hand, the present (cf. Fig. 4) and previous results obtained in rabbit neutrophils (2) show that secretion can be evoked without modifying the polar head group of PI. The inability of PDBu to stimulate incorporation of ^{32}P radioactivity into PI is indicative of an agent that is not involved in calcium "gating" (27). By contrast, formyl-Met-Leu-Phe, which enhances membrane permeability to calcium (1,6), elicits a marked increase in turnover of the polar head group indicative of a phospholipase-C mediated reaction (27).

In rabbit neutrophils, PDBu, like formyl-Met-Leu-Phe (3), induces a rather selective stimulation of arachidonate incorporation into PI. This may be caused by activating PI-specific phospholipase A_2, or may be a reflection of the relevant enrichment of arachidonic acid in PI, as also seen in unstimulated neutrophils. While arachidonic acid was also selectively incorporated into PI of unstimulated human neutrophils, the nonselectivity of PDBu-stimulated arachidonate incorporation into phospholipids of these cells could be interpreted in several ways. PDBu may stimulate a phospholipase A_2 that is not specific for PI in human neutrophils. The deacylation-recylation reaction induced by phorbol esters in canine kidney cells is catalyzed by a phospholipase A_2 that is not specific with regard to its phospholipid substrate (13, 28). Alternatively, cell activation by PDBu may

stimulate the transfer of arachidonic acid from rapidly turning over PA to phospholipids via acyltransferase. Indeed, the avidity with which PA incorporates arachidonic acid in human neutrophils may reflect a PA-specific deacylation-reacylation cycle that has previously been described in equine neutrophils (29) but is not present in rabbit neutrophils (Kramer and Rubin, unpublished observation). This would explain the difference in selectivity of arachidonyl-phospholipid turnover in rabbit and human neutrophils. These results also illustrate the caveats involved in comparing cellular mechanisms in cells of different species or types (recruited or resident neutrophils).

The present finding that PDBu, in contrast to calcium, was unable to activate phospholipase A_2 in broken cells (cf. Table 2), suggests that PDBu may be stimulating phospholipase A_2 by increasing calcium availability, rather than by directly stimulating the enzyme (11, 31). This conclusion is supported by the finding that the PDBu-induced increase in cell associated ^{45}Ca (cf. Fig. 5) paralleled the time-course of stimulated (arachidonyl)PI turnover and enzyme release. The inability of calcium deprivation to block completely PDBu-induced (arachidonyl)PI turnover suggests that extracellular calcium is not required for phospholipase A_2 activation in the neutrophil. Thus, cellular calcium may be used to activate phospholipase A_2 in the neutrophil, as in the exocrine pancreas (32) and platelet (33, 34). Arachidonate metabolites, synthesized as a consequence of phospholipase A_2 activation, may, in turn, serve as physiological calcium ionophores (35) and in this manner mediate several neutrophil functions, including degranulation (6,7,36). Although a close association between PDBu action and calcium is indicated by the present studies, the recently described similarity between the phorbol ester receptor and calcium/phospholipid-dependent protein kinase (37,38), as well as the ability of phorbol esters to stimulate this kinase (39), suggests that phorbol esters may act through an alternative mechanism.

Very recently, Sha'afi et al. (40) reported that a high concentration of TPA (1 μg/ml) stimulated calcium mobilization in rabbit neutrophils as monitored by quin-2 fluorescence. These investigators also observed that with a lower concentration of TPA (0.1 μg/ml), secretion was enhanced in the apparent absence of a rise in cytosolic ionized calcium. However, at the lower concentration, TPA was unable to elicit either a time- or dose-dependent stimulation of enzyme secretion; so, the relevance of these results to our own findings is uncertain. On the other hand, the recent findings of Serhan et al. (41), using human neutrophils, support the notion that an early response to phorbol esters is the release of membrane-associated calcium from intracellular stores.

In summary, the present account extends further the observation that stimulation of the deacylation-reacylation cycle represents a concomitant response to secretagogues, e.g., formyl-Met-Leu-Phe or ionophore A23187, which trigger calcium influx (6,42), and PDBu, which may mobilize cellular calcium (11,31). We cannot completely exclude the possibility that arachidonate turnover and enzyme secretion are parallel, but unrelated, events that occur during neutrophil activation by phorbol esters. However, the close association between this phospholipid effect and secretion, taken together with the increasing evidence for a role of arachidonate metabolites as mediators of neutrophil function (43,44), supports the contention that arachidonic acid turnover in position 2 of phospholipids is one modality by which signals for secretion are recognized and coupled to membrane events.

REFERENCES

1. Becker, E.L., Naccache, P.H., Showell, H.J., and Walenga, R.W. (1981) Lymphokines 4, 297-334.
2. Cockcroft, S., Bennett, J.P., and Gomperts, B.D. (1981) Biochem. J. 200, 501-508.
3. Rubin, R.P., Sink, L.E., and Freer, R.J. (1981) Mol. Pharmacol. 19, 31-37.
4. Rubin, R.P., Sink, L.E., and Freer, R.J. (1981) Biochem. J. 194, 497-505.
5. Rubin, R.P. (1982) Fed. Proc. Fed. Am. Soc. Exp. Biol. 41, 2181-2187.
6. Sha'afi, R.I., and Naccache, P.J. (1981) in Advances in Inflammation Research (Weissmann, G., ed.), Vol. 3, pp. 115-148, Raven Press, New York.
7. Stenson, W.F., and Parker, C.W. (1980) J. Immunol. 124, 2100-2104.
8. Goldstein, I.M., Horn, J.K., Kaplan, H.B., and Weissmann, G. (1974) Biochem. Biophys. Res. Commun. 60, 807-812.
9. Wright, D.G., Bralove, D.A., and Gallin, J.I. (1977) Amer. J. Pathol. 87, 273-284.
10. Smith, R.J., and Iden, S.S. (1979) Biochem. Biophys. Res. Commun. 91, 263-271.
11. Mottola, C., and Romeo, D. (1982) J. Cell. Biol. 93, 129-134.
12. Ohuchi, K., and Levine, L. (1978) J. Biol. Chem. 253, 4783-4790.
13. Beaudry, G.A., King, L., Daniel, L.W., and Waite, M. (1982) J. Biol. Chem. 257, 10973-10977.
14. Nakadate, T., Yamamoto, S., Ishii, M., and Kato, R. (1982) Cancer Res. 42, 2841-2845.
15. Hirata, F., Corcoran, B.A., Venkatsubramian, K., Schiffman, E., and Axelrod, J. (1979) Proc. Nat. Acad. Sci. (USA) 76, 2640-2643.
16. Driedger, P.E., and Blumberg, P.M. (1980) Proc. Natl. Acad. Sci. USA 77, 567-571.
17. Boyum, A. (1968) Scand. J. Clin. Lab. Invest. Suppl. 21, 77-89.

18. Bligh, E.G., and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911-917.
19. Weiss, S.J., McKinney, J.S., and Putney, J.W., Jr. (1982) Biochem. J. 204, 587-592.
20. Smolelis, A.N., and Hartsell, S.E. (1949) J. Bacteriol. 58, 731-736.
21. Wacker, W.E.C., Ulmer, D.D., and Vallee, B.L. (1956) N. Engl. J. Med. 255, 449-446.
22. Woollen, J.W., Heyworth, R., and Walker, P.G. (1961) Biochem. J. 78, 111-116.
23. Franson, R.C., Patriarca, P., and Elsbach, P. (1974) J. Lipid Res. 15, 380-388.
24. Poggioli, J., and Putney, J.W., Jr. (1982) Pflugers Arch. 392, 239-243.
25. Serhan, C.N., Broekman, M.J., Korchak, H.M., Marcus, A.J., and Weissmann, G. (1982) Biochem. Biophys. Res. Commun. 107, 951-958.
26. Walsh, C.E., Waite, M.B., Thomas, M.J., and DeChatelet, L.R. (1981) J. Biol. Chem. 256, 7228-7234.
27. Laychock, S.G., and Putney, J.W., Jr. (1982) in Cellular Regulation of Secretion and Release (Conn, P.M., ed.), pp. 55-105, Academic Press, New York.
28. Daniel, L.W., King, L., and Waite, M. (1981) J. Biol. Chem. 256, 12830-12835.
29. Lapetina, E.G., Billah, M.M., and Cuatrecasas, P. (1980) J. Biol. Chem. 255, 10966-10970.
30. Goodwin, B.J., and Weinberg, J.B. (1982) J. Clin. Invest. 70, 699-706.
31. Becker, E.L., Showell, H.J., Naccache, P.N., and Sha'afi, R.I. (1981) in Biochemistry of the Acute Allergic Reactions (Becker, E.L., Simon, A.S. and Austen, K.F., eds.) pp. 232-258, Alan R. Liss, New York.
32. Halenda, S.P., and Rubin, R.P. (1982) Biochem. J. 208, 713-721.
33. Pickett, W.C., Jesse, R.L., and Cohen, P. (1977) Biochim. Biophys. Acta 486, 209-213.
34. Van den Bosch, H. (1980) Biochim. Biophys. Acta 604, 191-246.
35. Naccache, P.H., Sha'afi, R.I., Borgeat, P., and Goetzl, E.J. (1981) J. Clin. Invest. 67, 1584-1587.
36. Serhan, C.N., Radin, A., Smolen, J.E., Korchak, H., Samuelsson, B., and Weissmann, G. (1982) Biochem. Biophys. Res. Commun. 107, 1006-1012.
37. Niedel, J.E., Kuhn, L.J., and Vandenbark, G.R. (1983) Proc. Natl. Acad. Sci. (USA) 80, 36-40.
38. Sando, J.J., and Young, M.C. (1983) Proc. Natl. Acad. Sci. (USA) 80, 2642-2646.
39. Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U., and Nishizuka, Y. (1982) J. Biol. Chem. 257:7847-7851.
40. Sha'afi, R.I., White, J.R., Molski, T.F.P., Shefcyk, J., Volpi, M., Naccache, P.H., and Feinstein, M.B. (1983) Biochem. Biophys. Res. Commun. 114, 638-645.
41. Serhan, C.N., Broekman, M.J., Korchak, H.M., Smolen, J.E., Marcus, A.J., and Weissmann, G. (1983) Biochim. Biophys. Acta 762, 420-428.
42. Smolen, J.E., and Weissmann, G. (1981) in Lysosomes and Lysosomal Storage Diseases (Callahan, J.W. and Lowden, J.A., eds.), pp. 31-62, Raven Press, New York.
43. Serhan, C.N., Fridovich, J., Goetzl, E.J., Dunham, P.B., and Weissmann, G. (1982) J. Biol. Chem. 257:4746-4752.
44. Sha'afi, R.I., Naccache, P.H., Molski, T.F.P., Borgeat, P., and Goetzl, E.J. (1981) J. Cell Physiol. 108:401-408.

Effect of Ingestion of Thermally Oxidized Frying Oil on Peroxidative Criteria in Rats

YAEKO IZAKI*[a], SHUNICHI YOSHIKAWA[a] and MITSURU UCHIYAMA[b], *Kyoto City Institute of Public Health[a], Kyoto, Japan, and National Institute of Hygenic Sciences[b], Tokyo, Japan*

ABSTRACT

Thermally oxidized rapeseed oils (4 levels of deterioration; used by a manufacturer of fried fish paste in a conventional manner) were fed to rats at a practical level of concentration. Rats were fed a diet ad libitum for 13 weeks that contained 15% of a test oil. The effects of the diet on several biochemical criteria related to peroxidative alterations were investigated.

In groups given thermally oxidized oils relative liver weight, relative kidney weight, thiobarbituric acid-reactive substances (TBA-RS) in the liver and reduced glutathione content were increased significantly in proportion to the degree of deterioration of the oil, compared with the group given fresh oil. Tocopherol contents in both serum and liver were decreased considerably in proportion to the deterioration level of the supplied oils.

The above criteria correlated well with various deterioration indices of the oil. For instance, TBA-RS was well correlated ($p<0.001$) with petroleum ether-insoluble oxidized fatty acid ($r=0.9191$), column chromatographically separated polar fraction ($r=0.9056$), glyceride dimer fraction ($r=0.9023$) and carbonyl value ($r=0.8647$).

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Because of the continued popularity of foods prepared by deep fat frying, establishing limitations on the extended usage of frying oils for both quality control and consumer protection has become important. Since Crampton et al. (1) first found toxic compounds in heated fats, some controversy has been generated regarding them. Reviews have been published by Nolen et al. (2), Mankel (3), Poling et al. (4), Waltking et al. (5) and Alexander (6). Broadly speaking, there are two schools of thought. One group of researchers (7,8) has isolated compounds from oils subjected to prolonged heating in the laboratory by vacuum distillation, urea adduct formation or column chromatography. They have observed pathological responses such as appetite and growth depression, diarrhea, histological changes in various tissues, and even death in some cases, after feeding these compounds to animals. This group takes a serious view regarding the potential toxicity of heated fats. The other group of researchers (2,4) has generally taken commercially used frying oils and either attempted to estimate the amount of the questionable components or fed the whole used fat to experimental animals as part of a balanced diet throughout the lifetime of the animals. The latter studies suggest that the suspected components are not present in ordinary commercially used oils in a significant quantity, and no deleterious effects were detected in the feeding studies.

To resolve the controversy, an experimental model that permits quantitative assessment of possible harmful effects of a heated fat was set up. For this purpose, we may use several biochemical criteria related to peroxidative alterations for detecting mild toxicity or low levels of toxic substances. According to Andia and Street (9), the ingestion of thermally oxidized oil increases endogeneous malonaldehyde. Peroxidative alterations have been identified as a basic deteriorative reaction (10) in the cellular mechanisms of aging and pathological disorders, and may be useful as a toxic response criterion at the biomembrane level.

In this study we attempted to define the influence of whole commercially used oils on the several biochemical criteria related to peroxidative deterioration, using rats fed a balanced diet containing the oils in a practical range of concentration.

*To whom correspondence should be addressed.

ABBREVIATIONS

ox.FA: Petroleum ether-insoluble oxidized fatty acid
CC-PF: Column chromatographically separated polar fraction
GD: Glyceride dimer fraction
TLC-UV_{233}: Thin-layer chromatography with ultraviolet detection at 233nm
P/Ap: Polar/apolar ratio measured by TLC-UV_{233}
RIT: Relative interface tension
TBA-RS: Thiobarbituric acid-reactive substance.

MATERIALS AND METHODS

Sample Oil Preparation

Thermally oxidized rapeseed oils were obtained from a manufacturer of fried fish paste (a traditional Japanese fried food named "Satsuma'age"). The fresh rapeseed oil (low erucic acid rapeseed oil containing citric acid at 20 ppm and silicone oil at 1.5 ppm, but not BHA or BHT; Yoshihara Seiyu Co., Ltd. Kobe, Japan) was used for frying fish

paste for a maximum of 66 days in an auto fryer (frying temperature, 180 C; heating time, 3.5 hr/day; capacity, 39.0 kg; surface area, 7,700 cm^2; fat turnover ratio, 4.1%/hr). Appropriate oil was added to the fryer at the end of each day to replace oil removed by sampling or adsorption by the fried fish paste.

Quality Assessment of Sample Oils

The quality of the frying oils was checked daily. Oils showing 4 levels of deterioration were used for animal feeding tests. The sample oils were kept in glass bottles under nitrogen at a temperature below 5 C. Carbonyl value (COV), peroxide value (POV), acid value (AV) and iodine value (IV) were measured by the standard methods of the Japanese Oil Chemists' Society (11). COV was represented as meq/kg for convenience in comparing it with other data. Petroleum ether-insoluble oxidized fatty acid (ox.FA) was determined by DGF-Einheitsmethoden C-III-3, rapid test (12). Column chromatographic separation of the polar fraction of frying oils has been proposed as a IUPAC-AOCS method (13). Glyceride dimer (GD) fraction, abundant in functional groups, was determined by silica gel column chromatography according to the method of Ohfuji and Kaneda (8). The polar/apolar (P/Ap) ratio was determined by the thin-layer chromatography with ultraviolet detection at 233nm (TLC-UV_{233}) method according to Urakami et al. (14). Relative interface tension (RIT) measurement has been proposed by Yoshikawa et al. (15) as a simple and convenient method to assess the quality of used frying oil. Tocopherols in oils were determined by a high-pressure liquid chromatography (HPLC) method with spectrofluorometric detection (16). The fatty acid composition of test oils were estimated by gas chromatography (GC) (11) using "Fat and oil reference mixtures No. 3" (Applied Science Lab., State College, PA) as a standard. C_{18}-Cyclic monomers were determined by GC according to the method of Meltzer et al. (17).

Animals and Treatments

Male COBS-Fisher (F-344/Ducrj) rats (Japan Charles River, Kanagawa, Japan), obtained at 4 weeks of age, were housed in individual wire cages. After one week for acclimation, rats weighing 98 to 108 g were randomly divided into 5 groups.

The basal diet [Oriental refined diet B with fat eliminated and selenium (0.5 ppm) added as sodium selenite] was purchased from Oriental Yeast Co. (Tokyo, Japan). The experimental diet also contained the selected amount (15%) of a test oil. The composition of the experimental diet is shown in Table 1.

The experimental diet was prepared once a week and stored in a closed container kept at a temperature below 5 C. Uneaten food was discarded every 3-4 days. Food and water were provided ad libitum, and food intake and body weight were measured every 3-4 days.

TABLE 1

Composition of Experimental Diet

Basal diet	
Cornstarch	34.4%
Milk casein	22.6%
Alpha starch	9.0%
Cellulose powder	7.2%
Granule sugar	4.5%
Vitamins[a]	1.8%
Minerals[b]	5.4%
Test oil	15.0%

[a]Vitamins (in 1 kg diet): retinyl acetate, 3.23 mg; cholecalciferol, 45.3 μg; thiamine HCl, 22 mg; riboflavine, 72 mg; pyridoxine HCl, 15 mg; cyanocobalamin, 90 μg; ascorbic acid, 540 mg; dl-α-tocopherol acetate, 90 mg; phylloquinone, 91 mg; biotin, 0.4 mg; folic acid, 3.6 mg; Ca-pantothenate, 90 mg; p-aminobenzoic acid, 90 mg; niacin, 90 mg; inositol, 109 mg; choline-Cl, 3.62 g.

[b]Minerals (in 1 kg diet): K, 4.01 g; P, 5.7 g; Ca, 4.31 g; Na, 1.97 g; Mg, 543 mg; Fe, 259 mg; Zn, 31 mg; Mn, 14 mg; Cu, 4 mg; I, 4 mg; Se, 0.5 mg.

Biological Procedures

At completion of the study (after 13 weeks of treatment with the experimental diet), the rats were killed by exsanguination under a light ether anesthesia. Blood was centrifuged at 2,500 rpm and thiobarbituric acid-reactive substance (TBA-RS) (18) and tocopherols (19) were estimated in the resultant serum.

The liver and kidneys were immediately excised, trimmed of connective tissues, and weighed. Samples of tissues (3 g) were weighed, added to 27 ml of cooled potassium phosphate buffer (75 mM, pH 7.0) and kept refrigerated. Homogenation and subcellular fractionation were done according to the method of Tsai et al. (20) as quickly as possible to avoid changes after excision. TBA-RS was determined by the method of Ohkawa et al. (21) and/or Uchiyama and Mihara (22), using 0.5 ml of 10% (w/v) whole homogenate, with tetraethoxypropane as a standard, and was expressed as malonaldehyde. Tissue-reduced glutathione (GSH) was determined by Ellman's method (23) with minor modifications.

After subcellular fractionation, the 100,000 g supernatant was assayed for glutathione peroxidase (GSH-px) and glutathione reductase (GR) activities. GSH-px activity was estimated by Little's method (24) using tertbutyl hydroperoxide as the substrate, which measures not only GSH-px activity but also GSH-S-transferases as well. GR activity was determined according to "Methods of Enzymatic Analysis" (25). Liver lipid was extracted with

TABLE 2

Characteristics of Test Oils

	Fresh	Heat-treated			
		A	B	C	D
Frying time (hr)	0	10.5	31.5	87.5	231.0
Carbonyl value (meq kg)	6.0	44.4	95.9	159.1	126.4
Peroxide value (meq kg)	1.1	5.0	8.9	7.6	5.3
Acid value	0.1	0.8	1.0	1.9	4.6
Iodine value	119.1	114.3	109.7	100.0	104.2
Polar material					
ox.FA (%)[a]	0.0	0.5	1.7	5.1	4.7
CC-PF (%)[b]	2.1	10.3	21.5	39.1	39.7
Glyceride dimer (%)[c]	0.0	0.6	1.0	2.8	2.3
P, Ap (TLC-UV$_{233}$)[d]	0.11	2.18	4.73	9.14	5.64
Viscosity (cp. 20 C)	99	117	155	270	281
Relative interface tension[e]	0.90	0.70	0.58	0.51	0.45
Tocopherols (μg g)	α.136.0				
	γ.367.5	0	0	0	0
	δ, 3.6				

[a]Petroleum ether-insoluble oxidized fatty acid.
[b]Polar fraction separated by silica gel column chromatography according to Waltking and Wessels (13).
[c]Glyceride dimer fraction obtained by silica gel column chromatography according to Ohfuji and Kaneda (8).
[d]Polar:apolar ratio measured by the TLC-UV method of Urakami et al. (14).

TABLE 3

Fatty Acid Composition of Test Oils[a]

	Fresh	Heat-treated			
		A	B	C	D
16:0	3.5	3.6	3.6	3.5	3.7
18:0	1.7	1.7	1.7	1.7	1.7
18:1	57.2	56.3	53.5	49.9	53.2
18:2	20.7	19.4	17.5	14.2	15.2
18:3	14.6	11.4	9.6	7.4	8.0
22:0	0.4	0.4	0.4	0.3	0.4
22:1	2.2	2.2	2.1	2.0	1.9
Noneluted material from GC	0.0	4.2	10.9	20.0	14.8

[a]Results are expressed as the percentage composition of fatty acid methyl esters determined by using Fat & Oil reference mixtures No. 3 (Applied Science Lab., State College, PA) as a standard.

chloroform/methanol (2:1, v/v) and determined gravimetrically. Liver lipid fatty acid composition was estimated by GC (11) and tocopherols were determined by HPLC (26).

RESULTS

Characteristics of Test Oils

Analytical values of the test oils are listed in Table 2, and the fatty acid composition of test oils is given in Table 3. COV, AV and the content of polar materials in each oil increased markedly during the frying period. The maximum values of COV and polar materials were obtained at 87.5 hr (heated time), while the AV and viscosity continued to increase with prolongation of the heating time. Some of the thermally oxidized materials were probably adsorbed from oil into the fried food between periods C and D. The fate of such materials should be traced by further experiments.

The fresh rapeseed oil was rich in oleic (57.2%), linoleic (20.7%) and linolenic (14.6%) acids. Erucic acid was present at a low level (2.2%). Decreases in the contents of unsaturated fatty acids were large during the frying period, especially linolenic acid. Noneluted material on GC represents polar substances in the fatty acid methyl esters; the amounts increased when the heating time was prolonged. C_{18}-Cyclic monomers were detected at low levels (fresh oil, 0%; thermally oxidized oil A, 0.04%; B, 0.13%; C, 0.22%; D, 0.18%).

The consistency of the test oils during the feeding study was confirmed by the determination of COV and POV each time a fresh batch of experimental diet was prepared; these values remained constant.

Fresh rapeseed oil contains natural tocopherols (α, 136 μg/g; γ, 368 μg/g; δ, 3.6 μg/g). Thermally oxidized oils contained no tocopherols. To determine whether added dl-α-tocopherol acetate was decomposed by contact with deteriorated oils or not, prepared diets were extracted with ether immediately and again 1 week after preparation,

TABLE 4

Results of Feeding Study[a]

Criteria	Control (n=8)	Heat-treated A (n=8)	Heat-treated B (n=5)	Heat-treated C (n=6)	Heat-treated D (n=6)	Commercial diet (n=7)
Body wt. gain (g)	201 ± 19	211 ± 12	205 ± 14	204 ± 9	218 ± 24	-
Food intake (g)	958 ± 72	1016 ± 60	974 ± 52	986 ± 35	1054 ± 82*	-
Feed efficiency[b]	0.210 ± 0.010	0.208 ± 0.010	0.210 ± 0.007	0.207 ± 0.005	0.206 ± 0.009	-
Relative liver wt. (%)	3.31 ± 0.12	3.39 ± 0.14	3.42 ± 0.11	3.74 ± 0.09**	3.47 ± 0.10*	3.08 ± 0.16
Relative kidney wt. (%)	0.60 ± 0.03	0.61 ± 0.02	0.64 ± 0.02**	0.70 ± 0.01**	0.68 ± 0.03**	0.55 ± 0.01

[a]Fresh rapeseed oil was used for the control diet. The characteristics of fresh oil and heat-treated oils (obtained from a manufacturer of fried fish paste) are given in Table 2. Rats were fed ad libitum for 13 weeks with diet which contained 15% of a test oil. Each value is the mean ± S.D. n=Number of rats examined.

[b]Feed efficiency=Body wt. gain/Food intake.

*Significantly different from control group, $p<0.05$.

**Significantly different from control group, $p<0.01$.

TABLE 5

Results of Feeding Study (Continued)

Criteria	Control (n=8)	Heat-treated A (n=8)	Heat-treated B (n=5)	Heat-treated C (n=6)	Heat-treated D (n=6)	Commercial diet (n=7)
TBA-reactive substance						
(MA, nmoles/g liver)[a]	210 ± 33	234 ± 32	277 ± 81	473 ± 43**	470 ± 58**	149 ± 19
(MA, nmoles/g liver)[b]	87 ± 8	93 ± 9	110 ± 20*	160 ± 13**	160 ± 22**	74 ± 7
(MA, nmoles/g kidney)[b]	110 ± 9	117 ± 9	109 ± 11	109 ± 5	102 ± 7	133 ± 17
(MA, nmoles/ml serum)[c]	3.7 ± 0.4	3.9 ± 0.6	3.4 ± 0.9	3.4 ± 0.3	3.2 ± 0.6	2.8 ± 0.7
GSH (μmoles/g liver)	1.06 ± 0.28	1.44 ± 0.28*	2.26 ± 0.59**	2.69 ± 0.52**	2.37 ± 0.44**	0.83 ± 0.06
GSH-px activity in liver[d]	105 ± 10	100 ± 7	84 ± 10**	88 ± 7**	80 ± 8**	116 ± 5
GSH-red activity in liver[d]	7.1 ± 2.4	6.1 ± 0.5	5.9 ± 0.9	6.4 ± 0.1	5.7 ± 0.9	-
Lipid content in liver (%)	4.8 ± 0.6	4.3 ± 0.5	4.7 ± 0.8	3.9 ± 0.3*	4.6 ± 0.5	4.6 ± 0.5
Tocopherol content						
(μg/g liver)	α,33.9 ± 2.8 γ,10.2 ± 1.9	22.1 ± 3.7**	15.9 ± 1.3**	7.7 ± 1.1**	8.9 ± 1.4**	42.5 ± 1.5
(μg/ml serum)	δ, 7.1 ± 1.0	5.8 ± 0.8	4.7 ± 0.4*	3.0 ± 0.3**	3.3 ± 0.2**	6.0 ± 0.4

[a]Measured by Ohkawa's method (21).

[b]Measured by Uchiyama's method (22).

[c]Measured by Yagi's method (18).

[d]μmoles NADPH oxidized/g liver/min.

and the extracts were saponified. Unsaponified materials were analyzed by HPLC. More than 96% of added dl-α-tocopherol acetate in all diets remained after 1 week.

Results of Feeding Study

Body weight gain, food intake, feed efficiency, relative liver weight and relative kidney weight are shown in Table 4.

Every diet was consumed in a normal manner. Food intake in the groups given heated oil was larger than that of the control group, but the body weight gain in all groups was essentially the same. No steatorrhea was observed throughout the feeding study. Significant differences were found in relative liver and kidney weight between the control group and groups given heated oil. The content of TBA-RS and other biochemical criteria are given in Table 5.

TBA-RS in liver homogenate, as determined by both Ohkawa's method (21) and Uchiyama's method (22), increased significantly in the groups given heated oil. TBA-RS in the kidney and serum were unchanged. Reduced GSH in the liver increased significantly in the groups given heated oil. GSH-px activity (including GSH-S-transferases) in the liver decreased significantly, while GR activity was unchanged.

Lipid content in the liver was unaffected except in group C. Tocopherols in the liver and serum were decreased considerably in proportion to the level of deterioration of the supplied oil. The fatty acid composition of liver lipid is given in Table 6. In the groups given heated oil, the relative amounts of palmitic (16:0), stearic (18:0), arachidonic (20:4) and docosahexaenoic (22:6) acids increased significantly, while oleic (18:1), linoleic (18:2) and linolenic (18:3) acids decreased.

DISCUSSION

There are a number of methods available for measuring lipid peroxidation in vivo and in vitro (e.g. determination of diene conjugation, TBA-RS, fluorescent products and exhalation of ethane and/or pentane in the breath). Among these methods, TBA assay has been widely used as a sensitive and simple method for animal tissues, although it is affected not only by the amount of malonaldehyde and/or lipoperoxides but also by the polyunsaturated fatty acids (PUFA) content, antioxidant level and iron catalyst in the tissue.

In many respects, lipid peroxidation and biomembranes are intimately related. Phospholipids composing the lipid bilayer of biomembranes are rich in PUFA, and oxidative attack results in the formation of PUFA radicals. These PUFA radicals absorb molecular oxygen to yield peroxy radicals and/or peroxides.

Lipid peroxides generated in biomembranes are bound to membranous protein. The combined peroxidative changes of several chemical species in fresh tissues homogenates can be measured by means of the TBA assay procedures proposed by Masugi and Nakamura (27), Ohkawa et al. (21) and Uchiyama et al. (22). Malonaldehyde precursors combined in lipid-protein complexes were measured by adding surfactant (21,27) or adjusting the pH (2.0) of the reaction mixture (22). According to

TABLE 6

Fatty Acid Composition of Liver Lipid[a]

	Control (n=3)	Heat treated A (n=3)	B (n=3)	C (n=3)	D (n=3)	Commercial diet (n=2)
14:0	0.5	0.4	0.3	0.3	0.4	0.5
16:0	17.7	18.3	17.6	18.5	20.5[b]	23.8
16:1	1.7	1.6	1.6	1.8	1.9	1.9
18:0	9.8	11.6	12.7[b]	13.5[c]	12.2[b]	10.3
18:1	34.9	32.5	31.7[c]	30.0[c]	32.8	13.5
18:2	13.2	11.7[b]	10.8[c]	9.2[c]	9.0[c]	21.1
18:3	3.0	2.3[b]	1.7[c]	1.3[c]	1.3[c]	1.2
20:3	0.6	0.6	0.7	0.6	0.6	1.1
20:4	11.1	13.0	14.8[b]	16.5[c]	13.9[b]	15.2
20:5	1.1	1.0	0.8[b]	0.8[b]	0.6[c]	0.8
22:4	1.1	1.1	1.0	1.0	0.8[b]	2.3
22:6	3.9	4.6[b]	4.9[c]	5.3[c]	4.7[b]	5.7

[a]Results are expressed as mean percentage composition of fatty acid methyl esters prepared from total liver lipids; n = number of animals examined.
[b]Significantly different from control group, $p<0.05$.
[c]Significantly different from control group, $p<0.01$.

Masugi and Nakamura (27) and Mihara et al. (28), the preexisting malonaldehyde level was rather low, and intact PUFA remained substantially unchanged while "injured" PUFA changed to TBA-RS. On the other hand, Matsushita et al. (29,30) studied the process of coloration in the TBA test of purified fatty acid monohydroperoxide. They demonstrated that metal salts, such as reduced iron, are useful for the release of TBA-RS from lipid hydroperoxide, and that oxygen is not needed for this reaction (31). They also suggested that prolonged heating in air with no addition of antioxidant could cause artificial autooxidation of coexisting native PUFA (32).

In this work, we used Ohkawa's (21) and/or Uchiyama's (22) methods. Since these methods require over 30 min for maximal color development for tissue homogenate, the possible occurrence of in vitro peroxidation could not be excluded. However, in vitro peroxidation during color development, as in the incubation of homogenate prior to TBA coloration, could be considered as the kind of reflection of peroxidative deterioration occurring in vivo.

The amounts of TBA-RS in the livers of rats of groups C and D were approximately twice that of the control. A good correlation was found between the results obtained by these two methods for fresh liver homogenate ($r=0.9770$, $p<0.001$).

Rats given the heated oil showed relative decreases of C-18 unsaturated fatty acids (18:1, 18:2, 18:3) and relative increases of arachidonic (20:4) and docosahexaenoic (22:6) acids in liver lipid. These increments in arachidonic and docosahexaenoic acid might possibly affect the results of TBA assay. However, in the group fed the commercial diet (Charles River CRF-1), in spite of the abundance of the PUFA, the TBA-RS level was very low. This result indicates indirectly that the rise of TBA-RS is not necessarily attributable to changes in the relative ratio of PUFA's in liver lipid or to artifactual formation during the reaction period.

That dietary vitamin E participates in lipid peroxidation in vivo (33) is well known. The experimental diet contained 90 IU/kg of vitamin E as dl-α-tocopherol acetate. Fresh oil contained α-tocopherol at a level of 136 μg/g, and it made up 15% of the diet, so the control group received about 120 IU/kg diet. The heated oils contained no tocopherol, so groups A-D were fed a 90 IU/kg diet. No decomposition of added dl-α-tocopherol acetate occurred in the experimental diet during the feeding test. For the normal rat or mouse, 15 IU/kg of vitamin E in the diet appears to be adequate (34). Though the vitamin E in the diet was more than sufficient in this experiment, the α-tocopherol content in the liver and serum decreased considerably as the deterioration level of the supplied oil was increased. The control group showed higher tocopherol levels in the liver and serum than the groups A-D. A graded decrease of hepatic and serum α-tocopherol levels was found in spite of the similar levels of dietary tocopherol in groups A-D, a fact of great importance. α-Tocopherol content and TBA-Rs in the liver were well correlated ($r=0.8542$, $p<0.001$.).

These observations suggest two possibilities: some oxidation products in heat-treated oil decomposed α-tocopherol in the gastrointestinal tract as the result of peroxidation in the intestinal lumen or they consumed α-tocopherol in liver biomembranes as the result of radical generation and subsequent lipid peroxidation. To clarify which actually occurs, the α-tocopherol absorption through intestinal tract with coexisting heated oil should be investigated further.

GSH is widely distributed in animal and plant cells. It plays an integral role in many biological functions, including the protection of cell membranes, the destruction of peroxides, the destruction of radiation-induced free radicals and the detoxification of xenobiotics. Wirth and Thorgeirsson (35) studied the synthesis and degradation of GSH in the rat liver by i.p. administration of diethylmaleate. They reported that liver GSH levels in normal adult rats were rapidly decreased to 15% of the control level 30 min after diethylmaleate administration and remained maximally depleted for 4 hr, after which they began to rise rapidly, returning to normal at 6 hr and to 200% normal at 24 hr. In our study, GSH levels in the livers of rats given heated oil were significantly increased, and GSH was probably consumed by a secondary oxidizing agent produced in the liver after the heated oils were ingested and an overshooting of resynthesis subsequently occurred. GSH and TBA-Rs in the liver were correlated ($r=0.6293$, $p<0.001$).

A "glutathione peroxidase system," consisting of GSH-px, GR and glucose-6-phosphate dehydrogenase, may function as a metabolic unit in the reduction of peroxides. Hepatic GSH-px (including GSH-S-transferases) activity was decreased in groups given heated oil, while GR activity was unchanged. In this study, experimental diets were supplemented with selenium (0.5 ppm). Reddy and Tappel (36) have shown that autooxidized lipid does not stimulate hepatic GSH-px when the diet is supplemented with selenium.

Since peroxide itself is normally present at low levels in heated fats, and its lymphatic absorption is very low, nonvolatile oxidized products accumulated during prolonged heating are important in assessing the biological effects of thermally oxidized oil. Combe et al. (37) reported that nonvolatile oxidized products, such as polymeric acids, oxidized monomeric acids and cyclic monomeric acids can be recovered from the lymphatic lipids. Accord-

ing to Artman and Smith (38), 136 components (0.42% of the fat) were separated from potato chips fried in cottonseed oil (182 C, 48 hr) and 51 components were characterized. Although describing the biological effects of heated fat with a single index might be difficult, the existing data from many feeding studies show that polar components, such as ox.FA, column chromatographically separated polar fraction (CC-PF), GD and cyclic monomers are the major factor responsible for the toxic effects.

On the basis of the linolenic acid content (14%) and heating conditions (180 C, in air), formation of C_{18}-cyclic monomers in this study was expected to be rather low, and was confirmed to be less than 0.22% by GC analysis. The German Society for Fat Research has recommended a level of 1.0% or more of ox.FA to aid sensory evaluation (39) in the quality assessment of used frying fats. Since the method of deterioration of ox.FA is time-consuming, a column chromatographic analysis was developed by Gupta (40) for the determination of polar components. Billek et al. (39) proposed that 1% of ox.FA corresponds to 27% (by weight) of polar components. On the other hand, Billek et al. (41) conducted feeding experiments with heated sunflower oil that had been used for the industrial production of fish fingers. The polar fraction was separated by means of column chromatography on silica gel, and then fed to rats at a concentration of 20% in the diet over a period of 18 months. Although the polar fraction caused a lower weight increase of the test animals, it showed no serious effects according to many biological, clinical and histological investigations. They calculated "a tentative ADI" of the polar fraction as 10 g/70 kg/day. Ohfuji et al. (42) proposed 1% GD formation in heated fats as an index of deterioration.

Using ox.FA, CC-PF and GD as indices of the quality of heated oils is reasonable, since good correlations ($p<0.001$) exist between these indices and TBA-RS (ox.FA, $r=0.9191$; CC-PF, $r=0.9056$; GD, $r=0.9023$). Though viscosity ($r=0.9205$) and IV ($r=-0.8796$) also showed good correlations with TBA-RS, these indices differ greatly in different sort of fats, and may not be suitable for adoption as general indices.

Since determining ox.FA, CC-PF and GD were very time-consuming, many simple and convenient methods have been developed. Urakami et al. (14) noticed that most of the components detected by Artman and Smith (38), and regarded as toxic, have a strong absorption at 233 nm, and they proposed a TLC-UV assay for assessment of the P/Ap ratio. P/Ap showed a good correlation with TBA-RS ($r=0.8296$). Relative interface tension (15) also showed a good correlation with TBA-RS ($r=-0.8123$).

The carbonyl value of heated oils is an accepted index of the degree of degradation from oxygen attack, and the high carbonyl values of the oxidized oils indicate that attack by molecular oxygen during heating produced considerable amounts of carbonyl materials. Uchiyama and Sato (43) pointed out that carbonyl compounds formed by the deterioration of oils, such as 12-keto oleic acid, have a prooxidant action on unsaturated fatty acids in vitro and, when ingested by animals, they might participate in lipid peroxidation in vivo. Budowski et al. (44) found that thermally oxidized safflower oil is very effective in producing nutritional encephalopathy when fed to young chicks in a diet deficient in vitamin E. These results suggest that unsaturated keto compounds formed in heated oil may cause lipid peroxidation in vivo. Carbonyl value also showed a good correlation with TBA-RS in the liver ($r=0.8647$).

According to Hemans et al. (45) and Iwaoka and Perkins (46), the results of this type of experiment depend on dietary protein level with the adverse effects of "used" fats progressively ameliorated by increasing levels of protein. Since the dietary protein level in this study was rather high (22.6%), food intake and body weight gain were found to be substantially the same in all groups. Even though the feeding study was carried out under very mild conditions, TBA-RS, GSH, vitamin E and the fatty acid profile clearly changed in proportion to the degradation indices of the supplied oils. These results suggest that some substances present in thermally oxidized oils act to promote peroxidative deterioration in the liver. We considered that the investigation of such criteria is a valuable experimental model for quantitative assessment of possible chronic effects of thermally oxidized oils. Further work is necessary to determine whether or not these results are universally applicable to used oils. Confirmation of the present results is also desirable by the use of other techniques, such as the measurement of diene conjugation, lipofoscin and/or exhalation of short-chain hydrocarbon gases in the breath.

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REFERENCES

1. Crampton, E.W., Common, R.H., Farmer, F.A., Wells, A.F., and Crawford, D. (1953) J. Nutr. 49, 333-346.
2. Nolen, G.A., Alexander, J.C., and Artman, N.R. (1967) J. Nutr. 93, 337-348.
3. Mankel, A. (1970) Fette Seifen Anstrichm. 72, 483-487.
4. Poling, C.E., Eagle, E., and Rice, E.E. (1970) Lipids 5, 128-136.
5. Waltking, A.E., Seery, W.E., and Bleffert, G.W. (1975) J. Am. Oil Chem. Soc. 52, 96-100.

6. Alexander, J.C. (1978) J. Am. Oil Chem. Soc. 55, 711-717.
7. Firestone, D., Horowitz, W., Friedman, L., and Shue, G.M. (1961) J. Am. Oil Chem. Soc. 38, 253-257.
8. Ohfuji, T., and Kaneda, T. (1973) Lipids 8, 353-359.
9. Andia, A.M.G., and Street, J.C. (1975) Agric. Food Chem. 23, 173-177.
10. Tappel, A.L. (1973) Fed. Proc. 32, 1870-1874.
11. Japanese Oil Chem. Soc. (1970) Standard Oils and Fats Analytical Methods, 2.4.22-73, 2.4.12-71, 2.4.1-71, 4.4.3-81, 2.4.20.2-77, 2.4.21.2-73.
12. Seher, A. (1963) Fette Seifen Anstrichm. 65, 1002-1004.
13. Waltking, A.E., and Wessels, H. (1981) J. Assoc. Off. Anal. Chem. 64, 1329-1330.
14. Urakami, C., Doi, H., Toriyama, S., Asano, Y., and Oka, S. (1976) Yukagaku 25, 764-772.
15. Yoshikawa, S., Izaki, Y., and Fujiwara, M. (1980) Yukagaku 29, 248-253.
16. Abe, K., Yuguchi, Y., and Katsui, G. (1975) J. Nutr. Sci. Vitaminol. 21, 183-188.
17. Meltzer, J.B., Frankel, E.N., Bessler, T.R., and Perkins, E.G. (1981) J. Am. Oil Chem. Soc. 58, 779-784.
18. Yagi, K. (1976) Biochem. Med. 15, 212-216.
19. Abe, K., and Katsui, G. (1975) Vitamins (Kyoto) 49, 259-263.
20. Tsai, A.C., Thie, G.M., and Lin, C.R. (1977) J. Nutr. 107, 310-319.
21. Ohkawa, H., Ohishi, N., and Yagi, K. (1979) Anal. Biochem. 95, 351-358.
22. Uchiyama, M., and Mihara, M. (1978) Anal. Biochem. 86, 271-278.
23. Ellmann, G.L. (1959) Arch. Biochem. Biophys. 82, 70-77.
24. Little, C., Olinescu, R., Reid, K.G., and O'Brien, P.J. (1970) J. Biol. Chem. 245, 3632-3636.
25. Bergmeyer, H.U. (1974) in Methods of Enzymatic Analysis, 2nd English edn., pp. 465-466, Academic Press, New York.
26. Abe, K., Ohmae, M., and Katsui, G. (1976) Vitamins (Kyoto) 50, 453-457.
27. Masugi, F., and Nakamura, T. (1977) Vitamins (Kyoto) 51, 21-29.
28. Mihara, M., Uchiyama, M., and Fukuzawa, K. (1980) Biochem. Med. 23, 302-311.
29. Asakawa, T., and Matsushita, S. (1980) Lipids 15, 137-140.
30. Terao, J., and Matsushita, S. (1981) Lipids 16, 98-101.
31. Asakawa, T., and Matsushita, S. (1981) Agric. Biol. Chem. 45, 453-457.
32. Asakawa, T., and Matsushita, S. (1979) Lipids 14, 401-406.
33. MaCay, P.B., Poyer, J.L., Pfeier, P.M., May, H.E., and Gilliam, K.M. (1971) Lipids 6, 297-306.
34. Bieri, J.G. (1972) Ann. N.Y. Acad. Sci. 203, 181-191.
35. Wirth, P.J., and Thorgeirsson, S.S. (1978) Cancer Res. 38, 2861-2865.
36. Reddy, K., and Tappel, A.L. (1974) J. Nutr. 104, 1069-1078.
37. Combe, N., Constantin, M.J., and Entressangles, B. (1981) Lipids 16, 8-14.
38. Artman, N.R., and Smith, D.E. (1972) J. Am. Oil Chem. Soc. 49, 318-326.
39. Billek, G., Guhr, G., and Waibel, T. (1978) J. Am. Oil Chem. Soc. 55, 728-733.
40. Gupta, A.K.S. (1976) Fette Seifen Anstrichm. 78, 111-118.
41. Billek, G., Guhr, G., and Sterner, W. (1979) Fette Seifen Anstrichm. 81, 562-566.
42. Ohfuji, T., Igarashi, H., and Kaneda, T. (1972) Yukagaku 21, 73-78.
43. Uchiyama, M., and Sato, M. (1970) J. Food Hyg. Soc. Jpn. 11, 327-333.
44. Budowski, P., Bartov, I., Dror, Y., and Frankel, E.N. (1979) Lipids 14, 768-772.
45. Hemans, C., Kummerow, F., and Perkins, E.G. (1973) J. Nutr. 103, 1665-1672.
46. Iwaoka, W.T., and Perkins, E.G. (1976) Lipids 11, 349-353.

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Fatty Acid Metabolism in Young Oysters, *Crassostrea gigas:* Polyunsaturated Fatty Acids

M.J. WALDOCK[1] and **D.L. HOLLAND,*** *N.E.R.C. Unit of Marine Invertebrate Biology, Marine Science Laboratories, Menai Bridge, Anglesey LL59 5EH, United Kingdom*

ABSTRACT

Tetraselmis suecica and *Dunaliella tertiolecta* were grown for 24 hr in the presence of ^{14}C sodium bicarbonate and then fed separately to batches of juvenile oysters, *Crassostrea gigas,* for 3 days. *D. tertiolecta* contained fatty acids no longer than C_{18}; 22:6ω3 was absent in *T. suecica.* Analysis of the oyster fatty acids by radio gas chromatography (GC) showed that oysters were able to incorporate some of the dietary ^{14}C label into long-chain fatty acids not supplied in the diet, e.g., C_{20} and C_{22} mono- and polyunsaturated fatty acids, and particularly 20:5ω3. However, the low ^{14}C incorporation into fatty acids longer or more unsaturated than those supplied in the diet suggests that elongation and desaturation activity in young oysters is not sufficient to sustain optimum growth.
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INTRODUCTION

Dietary ω3 long-chain polyunsaturated fatty acids (PUFA), e.g., 20:5ω3 and 22:6ω3, are essential for the optimum growth of several marine fish (1-4), and for penaeid prawns (5-9). However, freshwater fish, e.g., rainbow trout and carp, can biosynthesize the long-chain C_{22} PUFA from shorter chain precursors and may be cultured successfully on diets containing C_{18} ω3 and ω6 fatty acids (10-12). Further studies on fish and prawns (13,14) have confirmed that many freshwater species have active fatty acid elongation and desaturation systems, whereas marine species, especially those high in the food chain, e.g., turbot and plaice (13), have a reduced ability to synthesize, from shorter chain precursors, the important C_{22} ω3 long-chain fatty acids by desaturation and elongation.

In a series of studies on the bivalve clam, *Mesoderma mactroides,* Moreno et al. (15,16) have shown that linoleic acids, 18:2ω6, and α-linolenic acid, 18:3ω3, can be desaturated and to some extent elongated to higher homologues, e.g., 20:2ω6, 18:4ω3 and 20:3ω3, but no biosynthesis of the longer and more unsaturated acids such as 20:4ω6, 20:5ω3 and 22:6ω3 occurred.

In this laboratory, the studies on juveniles of another bivalve, the Pacific oyster, *Crassostrea gigas,* have shown that dietary requirements exist for ω3 long-chain PUFA when animals are actively growing (17). Thus, when 20:5ω3 and 22:6ω3 were absent in the algal diets, the growth of juvenile oysters was poor and endogenous PUFA were rapidly depleted. Growth was improved when the algal diets were supplemented with microencapsulated ω3 C_{22} PUFA. Whether or not the ability to elongate and desaturate dietary C_{18} fatty acids was low or totally absent was not conclusive.

Because bivalves can directly incorporate all labeled algal fatty acids into tissue lipids (15) and because, in this laboratory, routine cultures of *Dunaliella tertiolecta* contain fatty acids no longer or more unsaturated than 18:3ω3 and cultures of *Tetraselmis suecica* contain no trace of 22:6ω3, this provided the basis for an in vivo study on the capability of young oysters to synthesize C_{20} and C_{22} ω3 PUFA from dietary algal fatty acids. *T. suecica* and *D. tertiolecta*, preincubated with ^{14}C bicarbonate to label the fatty acids, were fed to juvenile *Crassostrea gigas* at a stage when young oysters are still actively accumulating lipid in the body tissues (18) and the incorporation of radiolabel into tissue fatty acids was determined by radio gas chromatography (GC).

MATERIALS AND METHODS

Materials

^{14}C labeled sodium bicarbonate was obtained from Amersham International, Amersham, U.K.

Labeling Algal Fatty Acids

Nonaxenic cultures of *D. tertiolecta* and *T. suecica* were grown in 20 l borosilicate flasks under continuous illumination (from 10,000 lux, daylight fluorescent lamps at 18 C) in filtered seawater, salinity 32‰, enriched with Conway medium (19). The cultures were gently agitated by aeration and harvested during the growth phase on a semicontinuous basis.

[1] Present address: M.A.F.F. Fisheries Laboratory, Burnham-on-Crouch, Essex CMO 8HA, U.K.

Two hundred ml of each algal culture were taken during the growth phase and placed in 2 l conical flasks containing 200 ml filtered, UV-irradiated seawater, salinity 32‰, enriched with Conway medium, and 100 μCi of ^{14}C sodium bicarbonate was added to the flask and mixed by gentle swirling. The algae were cultured at 15 C under continuous illumination from 10,000 lux daylight fluorescent lamps. Each culture was left for 24 hr with occasional swirling and then fed to the spat. Samples of the labeled algae were filtered off and taken to determine the level of ^{14}C incorporation by scintillation counting.

Culture and ^{14}C Labeling of C. gigas Juveniles

Young oysters were provided by the Fisheries Experiment Station, Conway, N. Wales, U.K. In 2 separate experiments, 2 batches of young oysters were maintained in filtered and UV-irradiated sea water at a salinity of 25‰ and at 24 C ± 1 C. One batch was fed on nonlabeled *D. tertiolecta* and the other on *T. suecica* for 1 week before the labeling experiments. Algae were added at a concentration of 10^6 cells per mg of live fresh weight of oyster per week as described by Walne (20). The spat were fed twice daily and the water changed every other day. The cultures were agitated by gentle aeration (100 ml filtered air/min) to keep the algal cells in suspension. The batches of oysters were then fed on the appropriate single species of radiolabeled algae in the same way for 3 days, followed by 1 day of feeding on nonlabeled algae, to ensure that all the radiolabel was absorbed from the gut and incorporated into the body tissues. Finally, the oysters were starved for 24 hr to clear the gut of any algae. Samples of oysters were then taken from each batch, briefly washed in chloroform/methanol (2:1, v/v) to remove lipids from any attached algae on the outside of the shell and digested in a N.C.S. (Amersham International, U.K.) tissue solubilizer. The total incorporated ^{14}C radioactivity was then determined by scintillation counting using a Tracerlab Services scintillation counter.

The remainder of the batches were briefly washed in chloroform/methanol (2:1, v/v) and then homogenized for 3 min in a minimum volume of seawater in a top-drive macerator. Total lipid was extracted according to the procedure of Folch et al. (21) with 20 vol chloroform/methanol (2:1, v/v) containing 0.005% w/v of the antioxidant 2,6-di-tert-butyl-p-cresol, and finally stored in chloroform/antioxidant. The extracted lipids were then methylated with BF_3 and methanol (22). Impurities were removed from the fatty acid methyl esters by thin layer chromatography (TLC) on Merck precoated silica gel plates in a continuous elution system. Plates were developed for 90 min with petroleum ether (60-80 C)/diethyl ether/glacial acetic acid (85:15:1) at room temperature (ca. 22 C). Separation of the methyl esters and determinaton of ^{14}C incorporation was carried out on a Pye 104 gas liquid chromatograph coupled to an E.S.I. Nuclear radio gas detector. The conventional 5′ × ¼″ i.d. glass column was packed with 5% SP 1000 on Chromasorb W, AW DMCS 80-100 mesh. Argon was used as the carrier gas and the temperature program was 130-200 C at 2 C/min. Analysis of nonlabeled methyl esters was carried out using a Carlo Erba 4160 fitted with a 20 m × 0.5 mm i.d. glass capillary column coated with SP 1000. Hydrogen was used as the carrier gas and the temperature program was 130-200 C at 2 C/min.

RESULTS AND DISCUSSION

The levels of incorporation of the ^{14}C radiolabel into the dietary algae and into oyster lipid is shown in Table 1. After 3 days, ca. 10% of the ^{14}C activity in oyster tissues was in the lipid fraction.

Table 2 shows the distribution of ^{14}C label

TABLE 1

Assimilation of Radioactivity from ^{14}C Labeled *D. tertiolecta* and *T. suecica* into *C. gigas* Spat

Diet	Experiment	Total ^{14}C in the diet (dpm)	Total ^{14}C recovered in each spat (dpm)	^{14}C in oyster lipid (dpm)	Percent of total ^{14}C label incorporated by the oysters as lipid
D. tertiolecta	1	4.06×10^6	3.98×10^5	4.34×10^4	10.90
	2	6.30×10^5	1.56×10^4	1.33×10^3	8.52
T. suecica	1	6.90×10^6	2.68×10^6	2.75×10^5	10.26
	2	9.59×10^5	1.55×10^5	2.04×10^4	13.20

TABLE 2

Labeling Distribution in *C. gigas* Fatty Acids after Feeding for 3 Days on a Single Species of ^{14}C Labeled Algae

Diet	Experiment	Major fatty acid or group of fatty acids											
		14:0 14:1	16:0 16:1	16:2 16:3 16:4	18:0 18:1	18:2 18:3 18:4	20:1 20:2	20:3 20:4	20:5ω3	22:1	22:2 22:3 22:4	22:5ω3	22:6ω3
D. tertiolecta	1	3.9	21.7	11.0	19.8	28.0	6.2	6.7	0.5	1.6	nd	nd	0.3
	2	6.0	24.2	1.8	20.7	26.1	7.0	6.7	0.9	4.3	nd	nd	0.9
T. suecica	1	0.5	19.8	6.9	17.2	18.1	10.4	2.6	14.5	5.7	2.2	0.9	nd
	2	2.1	20.1	2.5	13.7	16.4	13.4	2.9	24.8	0.5	1.0	1.2	0.5

nd = not detected.
The results are expressed as the percentage of total recovered ^{14}C radiolabeled fatty acids.

TABLE 3

Fatty Acid Composition of Total Lipids of *D. tertiolecta* and *T. suecica*

Fatty acids	*D. tertiolecta*	*T. suecica*
14:0	2.1	4.2
14:1	0.2	trace
15:0	0.7	0.5
16:0	18.4	21.9
16:1ω9	0.3	2.2
16:1ω7	1.8	2.0
16:1ω5	0.5	nd
16:2ω6	1.3	0.3
16:3ω6	1.0	trace
16:3ω3	3.1	0.7
16:4ω3	13.1	13.1
17:0	0.5	nd
18:0	0.9	2.2
18:1ω9	5.0	20.8
18:1ω7	0.6	trace
18:1ω5	trace	nd
18:2ω6	7.1	1.6
18:3ω6	4.0	trace
18:3ω3	36.2	8.2
18:4ω3	nd	3.1
20:1	nd	3.0
20:2ω6	nd	trace
20:3ω3	nd	nd
20:4ω6	nd	0.8
20:4ω3	nd	1.0
20:5ω3	nd	12.6
22:1ω11+ω9+ω7	nd	nd
22:3ω3	nd	nd
22:4ω6	nd	nd
22:5ω3	nd	nd
22:6ω3	nd	nd

nd = not detected.
The results are expressed as the percentage of total fatty acids present.

in the fatty acids of juvenile oysters after feeding ^{14}C labeled *D. tertiolecta* or *T. suecica* for 3 days.

The highest proportion of ^{14}C in oyster fatty acids was in fatty acids that are also present in the dietary algae (Table 3) and were presumably incorporated directly into the lipid reserves of the oysters in a largely unaltered form, as dietary algal lipids are in the clam *M. mactroides* (15). In oysters fed *D. tertiolecta*, 84.4% and 78.8% of the ^{14}C label in fatty acids were recovered in fatty acids less than C_{18} in chain length, and only 18.4% and 15.3% in the fatty acids longer or more unsaturated than C_{18}. In oysters fed *T. suecica*, 91.3% and 98.9% of the label in fatty acids were present in fatty acids up to 20:5ω3 in chain length. Longer chain fatty acids contained only 8.8% and 3.2% of the ^{14}C label. Because *D. tertiolecta* contains fatty acids no longer or more unsaturated than 18:3ω3 and *T. suecica* contains fatty acids no longer or more unsaturated than 20:5ω3

(Table 3), the ^{14}C label in the C_{20} and C_{22} fatty acids in oysters fed *D. tertiolecta* and the C_{22} fatty acids in oysters fed *T. suecica* must have been formed by modifying dietary precursor fatty acids within the tissues of the oysters or by de novo synthesis in the case of nonessential fatty acids. Chain elongation could account for the ^{14}C label in the nondietary C_{20} and C_{22} fatty acids with the exception of 20:5ω3 and 22:6ω3 in oysters fed *D. tertiolecta* and 22:6ω3 in oysters fed *T. suecica*. Although elongation of 20:5ω3 could account for the label in 22:5ω3 in oysters fed *T. suecica*, elongation cannot account for the ^{14}C label in 20:5ω3 or 22:6ω3 when these acids are absent in the diet as a desaturation step is necessary in the biosynthesis. Less than 1% of the ^{14}C label was found in 20:5ω3 and 22:6ω3 when these fatty acids were omitted from the diet, indicating that desaturation activity is low. The improvement of oyster growth with the addition of PUFA such as 20:5ω3 and 22:6ω3 in the diet (17) does suggest that the activities of desaturation enzymes in the oyster are too low to sustain optimum growth. Young oysters grow much better on a diet of *T. suecica*, which contains appreciable levels of the PUFA 20:5ω3, one of the ω3 PUFA so important in marine animals, than on *D. tertiolecta* (17), which contains no trace of the ω3 C_{20} or C_{22} PUFA. The fatty acid composition of the algae is shown in Table 3.

In mature oysters, *Crassostrea virginica*, Trider and Castell (23) found that better weight gain was achieved by feeding cod-liver oil containing ω3 fatty acids than by feeding ω6 fatty acids in the form of corn oil and concluded that oysters appear to have a major dietary requirement for ω3, and possibly a minor requirement for ω6, PUFA. However, they did not indicate whether or not these fatty acids should be C_{18} or C_{20}/C_{22} PUFA.

In Moreno's experiments with copepods (24), after 5 hr incubation with [1-^{14}C] α-linolenic acid, the ^{14}C label in 20:5ω3 and 22:6ω3 was only 1.9% and 0.6%, respectively, of the total label recovered in the fatty acids; other ω3 PUFA contained significantly less than 1% of the ^{14}C label. These figures are comparable with those in Table 2 for ^{14}C labeled 20:5ω3 and 22:6ω3 in oysters fed *D. tertiolecta*, i.e., synthesized from shorter chain precursors. On the other hand, in the clam, *M. mactroides*, Moreno et al. (15) detected no ^{14}C activity at all in 20:5ω3 or 22:6ω3 after administering 18:3ω3 for 12 hr. In Moreno's experiments, the clams were placed in a synthetic seawater medium containing dissolved radiolabeled [1-^{14}C] linoleic and [1-^{14}C] α-linolenic acid, supplying the ^{14}C label directly in the diet for 72 hr as in the experiments described in this paper, may provide a reason for the differences between clams and oysters. Nevertheless, the oyster, *C. gigas*, has some ability to elongate and desaturate ω3 fatty acids to produce 20:5ω3 and 22:6ω3, whereas this activity is lacking in the clam, *M. mactroides*. All this points the way for further studies on fatty acid desaturase and elongation enzymes in bivalves and marine invertebrates in general.

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REFERENCES

1. Yone, Y., and Fujii, M. (1975) Bull. Jpn. Soc. Sci. Fish. 41, 73-77.
2. Cowey, C.B., Adron, J.W., Owen, J.M., and Roberts, R.J. (1976) Comp. Biochem. Physiol. 53B, 399-403.
3. Cowey, C.B., Owen, J.M., Adron, J.W., and Middleton, C. (1976) Br. J. J. Nutr. 36, 479-486.
4. Fujii, M., and Yone, Y. (1976) Bull. Jpn. Soc. Sci. Fish. 42, 583-588.
5. Colvin, P.M. (1976) Aquaculture 8, 81-89.
6. Guary, J.C., Kayama, M., Murakami, Y., and Ceccaldi, H. (1976) Aquaculture 7, 245-254.
7. Kanazawa, A., Teshima, S., and Tokiwa, S. (1977) Bull. Jpn. Soc. Sci. Fish. 43, 849-856.
8. Kanazawa, A., Tokiwa, S., and Kayama, M. (1977) Bull. Jpn. Sci. Fish. 43, 1111-1114.
9. Jones, D.A., Kanazawa, A., and Ono, D. (1979) Mar. Biol. 54, 261-267.
10. Castell, J.D., Sinnhuber, R.O., Wales, J.H., and Lee, D.J. (1972) J. Nutr. 102, 77-86.
11. Watanabe, T., Ogino, C., Koshüshi, Y., and Matsunaga, T. (1974) Bull. Jpn. Soc. Sci. Fish. 40, 493-499.
12. Watanabe, T., Utsue, D., Kobayashi, I., and Ogino, C. (1975) Bull. Jpn. Soc. Sci. Fish. 48, 257-262.
13. Owen, J.M., Adron, J.W., Middleton, C., and Cowey, C.B. (1975) Lipids 10, 528-531.
14. Kanazawa, A., Teshima, S., and Ono, K. (1979) Comp. Biochem. Physiol. 63B, 295-298.
15. de Moreno, J.E.A., Moreno, V.J., and Brenner, R.R. (1976) Lipids 11, 561-566.
16. de Moreno, J.E.A., Moreno, V.J., and Brenner, R.R. (1977) Lipids 12, 804-808.
17. Langdon, C.J., and Waldock, M.J. (1981) J. Mar. Biol. Assoc. U.K. 61, 431-448.
18. Holland, D.L. (1978) in Biochemical and Biophysical Perspectives in Marine Biology (Malins, D.C., and Dargent, J.R., eds.) Vol. 4, pp. 85-123, Academic Press, London.
19. Walne, P.R. (1966) Fish. Invest. Ser. London II 25(4).
20. Walne, P.R. (1970) Fish. Invest. Ser. London II 26(5).

21. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497-507.
22. Morrison, W.R., and Smith, L.H. (1964) J. Lipid Res. 5, 600-608.
23. Trider, D.J., and Castell, J.D. (1980) J. Nutr. 110, 1303-1309.
24. Moreno, V.J., de Moreno, J.E.A., and Brenner, R.R. (1979) Lipids 14, 313-317.

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Distribution of the Molecular Species of Phospholipids in Human Umbilical Cord Blood

W. CARL BRECKENRIDGE*[a], PETER J. DOLPHIN[a] *and* MENG H. TAN[b], *Departments of [a]Biochemistry and [b]Medicine, Dalhousie University, Halifax, Nova Scotia B3H 4H7, Canada*

ABSTRACT

The composition of phosphatidylcholine (PC) and sphingomyelin (SM) was studied in cord blood lipoproteins to determine whether equilibration of the molecular species of phospholipids among lipoproteins was comparable with that reported for adults. The molecular species distributions of PC in low density lipoprotein (LDL) differed from that of high density lipoprotein (HDL). Whereas LDL PC was richer in combinations of fatty acids with 16 and 18 carbon atoms than HDL, the HDL was markedly enriched in combinations of fatty acids with 18 and 20 carbon atoms. Sphingomyelins in LDL were richer in palmitic acid than HDL while HDL had a greater proportion of long chain sphingomyelin than LDL. The molecular species of PC and SM do not equilibrate in cord blood. The results for the SM distributions were similar to other reports for adult human lipoprotein. However, the marked differential distribution of PC among lipoproteins appears unique to cord blood. The mechanisms responsible for equilibrating PC among lipoproteins are less well developed in the neonate when compared with the adult.
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INTRODUCTION

The equilibration of phospholipids (PL) among adult plasma lipoproteins appears to occur by 2 possible mechanisms. During the catabolism of triacylglycerol-rich lipoproteins, a portion of the PL is transfered to the HDL fraction (1-3). PL also exchange among plasma lipoproteins (4). Both processes appear to be stimulated by proteins in plasma (5,6). Thus, all molecular species of PL are thought to equilibrate among plasma lipoproteins. Recently, the molecular species composition of respective PL have been shown not to be equal in lipoproteins from adult plasma (7-9). Furthermore, inhibition of lecithin-cholesterol acyl transferase (LCAT) activity reduces the exchange of sphingomyelin (SM) but does not affect the rate of phosphatidylcholine (PC) exchange (4).

The lipid transport processes in the neonate are different from the adult human where active fat transport from the intestine occurs. Although LCAT activity is reduced in cord blood when compared with the adult (10), no reports concerning the presence of exchange proteins have been made. Furthermore, little data is available concerning the composition of the molecular species of PL in cord blood lipoproteins. Therefore, determining whether the composition of the molecular species of phospholipids in cord blood lipoproteins is comparable with that of adult plasma is interesting as a prerequisite to assessing whether this process is altered when the newborn begins to consume diets of varying fat content. The data indicate that a much greater difference in the composition of the molecular species of PC of cord blood LDL and HDL exists when compared with that of adult lipoproteins.

*To whom correspondence should be addressed.

EXPERIMENTAL

Patients

Umbilical cord blood samples were obtained at delivery from healthy, normal birth, term human foetae of mean gestation 39.9 ± 1.6 weeks with Apgar scores of greater than 9 at 5 min. The samples were maintained at 4 C and within 12 hr the serum was recovered by low-speed centrifugation. Thimerosal (Sigma Chemical Co., St. Louis, MO) was added to a final concentration of 0.1% and the sera were then screened for maternal blood contamination as indicated by the presence of IgA on Ouchterlony radial diffusion analysis using Hyland immunoplates containing antihuman IgA (Hyland Diagnostics, Deerfield, IL) as previously described (11). Twelve percent of all cord sera received (N = 171) contained detectable quantities of IgA and were rejected for further analysis. This study was subjected to ethical review by the Faculty of Medicine, the maternity hospital concerned and the granting agency.

MATERIALS AND METHODS

Lipoprotein Isolation

Lipoproteins were isolated by sequential

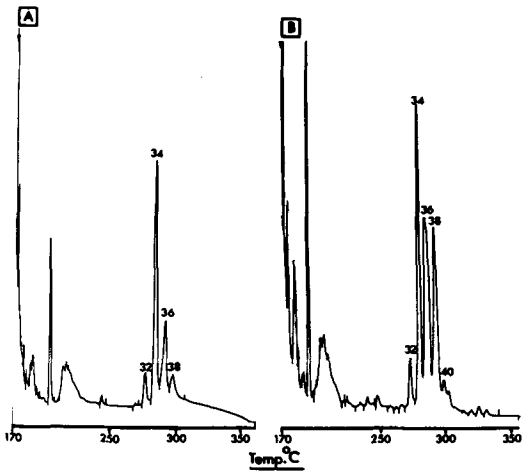

FIG. 1. GLC of t-butyldimethylsilyl ethers of the diacylglycerols from PC of cord blood lipoproteins; A–LDL; B–HDL_3. Carbon numbers identify the number of fatty acid carbons in the diacylglycerol component of the PC.

ultracentrifugation in a Beckman L5-50 ultracentrifuge using a 50.3 Ti rotor at the following densities, VLDL d=1.006 kg/l for 1.10×10^8 g/min; LDL, d=1.063 kg/l for 1.44×10^8 g/min; HDL_2 d=1.125 kg/l for 1.57×10^8 g/min and HDL_3 d=1.21 kg/l for 1.71×10^8 g/min.

Lipid Analysis

Phospholipids were extracted by the method of Folch et al. (12). SM and PC were separated by thin layer chromatography (TLC) on silica gel G using a solvent system of chloroform/methanol/acetic acid/water (74:45:12:6). The lipids were recovered from the silica by extraction with a mixture of chloroform/methanol/acetic acid/water (50:39:10:1), and digested with phospholipase C as described by Kuksis et al. (13). The fractions were purified on silica gel using chloroform/methanol (90:10) as a solvent. Following recovery, the ceramides were converted to t-butyldimethylsilyl ether derivatives (7). Diacylglycerols were analyzed as the trimethylsilyl or t-butyldimethylsilyl ethers. The molecular species of diacylglycerols or ceramides were resolved according to molecular weight by gas liquid chromatography (GLC) on 3% OV-1 (Applied Science Labs., State College, PA). Identification of carbon numbers was based on previous work on human PL (7,9). Fatty acids of isolated PL were converted to methyl esters and analyzed (14) by GLC on 6-ft stainless-steel columns packed with 10% EGSS-X (Applied Science Labs.).

TABLE 1

Molecular Species of Diacylglycerols from Phosphatidylcholines of Cord Blood Lipoproteins*

Carbon† number	Lipoprotein‡ (wt %)		
	LDL	HDL_2	HDL_3
C32	4.8 ± 2.0	2.1 ± 2.5	2.0 ± 1.0
C34	34.8 ± 10.0[a,b]	23.2 ± 6.8§	22.5 ± 2.2[a]
C36	31.4 ± 3.1	32.5 ± 3.6	34.0 ± 3.4
C38	21.3 ± 6.1[a]	35.8 ± 6.2[a]	31.8 ± 3.7[a]
C40	6.5 ± 4.5	8.8 ± 5.4	8.9 ± 3.0

Values for a particular carbon number in different lipoproteins are significantly different where labelled: a) $p < 0.001$; b) $p < 0.02$.

*Results are the mean ± SD of 8 samples.

†Carbon number represents number of fatty acyl carbon atoms in the diacylglycerol.

§Density fractions for isolation were LDL = 1.006-1.063, HDL_2 = 1.063-1.125 and HDL_3 = 1.125-1.21 g/ml.

RESULTS

Initial studies of the total lipid profiles of cord blood established that good recovery of PL by the phospholipidase C digestion (11) occurred. The data also indicated that the proportion of longer chain molecular species of PL in cord blood was greater than usually noted for adult serum (8,9). Following isolation of the PC and conversion to the diacylglycerols, we observed (Fig. 1) that a marked difference existed between the molecular species profile for the LDL and HDL fractions. The LDL was characterized by a major component corresponding to fatty acid combinations of C16 and C18 (C34) fatty acids. Much lower amounts of of carbon number C36 and C38 were found, which represent combinations of C16 and C18 fatty acids with C20 fatty acids. The predominance of the C34 species over the other carbon numbers was most variable in LDL however. High density lipoproteins had approximately equal amounts of C34, C36 and C38 molecular species. The results of the molecular species analysis are shown in Table 1. The LDL had significantly higher amounts of C34 and significantly lower amounts of C38 than with HDL_2 or HDL_3 whereas the proportion of C36 was approximately equal in both groups. The reasons for these differences were apparent from the fatty acid composition of the PC (Table 2). The C20 unsaturated fatty acids accounted for 23% of the fatty acids in HDL_3 but only 12% in LDL. The proportion of palmitic and stearic acids remained relatively constant in both lipoproteins.

The SM composition was also distinct for

TABLE 2

Fatty Acid Composition of Phospholipids from Cord Blood Lipoproteins[a]

Fatty acid[b]	PC (wt %)		SM (wt %)	
	LDL	HDL_3	LDL	HDL_3
16:0	42.3 ± 1.8	38.6 ± 3.3	33.0 ± 2.1	21.5 ± 2.1
16:1	0.9 ± 0.3	0.7 ± 0.3	1.1 ± 0.4	1.0 ± 0.5
18:0	18.9 ± 0.8	18.7 ± 1.1	15.4 ± 0.9	13.0 ± 1.1
18:1	14.2 ± 0.9	11.9 ± 1.3	1.5 ± 0.5	1.1 ± 0.8
18:2	9.5 ± 0.5	5.6 ± 1.1	—	—
20:0	—	—	9.3 ± 1.0	7.5 ± 0.9
20:1	—	—	tr	—
20:2	0.5 ± 0.5	3.4 ± 0.9	—	—
20:3	2.4 ± 0.6	5.0 ± 1.0	—	—
20:4	10.0 ± 1.1	15.1 ± 1.4	—	—
22:0	—	—	11.2 ± 0.8	14.1 ± 1.2
22:6	1.2 ± 0.4	1.1 ± 0.4	—	—
24:0	—	—	7.1 ± 0.9	11.1 ± 1.3
24:1	—	—	21.1 ± 2.3	30.1 ± 2.2

[a]Results are mean ± SD of analyses from 3 separate lipoprotein preparations.
[b]Fatty acid identified by number of carbon atoms and double bonds.

TABLE 3

Molecular Species of Ceramides from Sphingomyelins of Cord Blood Lipoproteins*

Carbon number†	Lipoprotein‡		
	LDL	HDL_2	HDL_3
C34	30.4 ± 6.9[a]	28.5 ± 2.0	20.9 ± 3.0[a]
C36	15.8 ± 1.8	15.8 ± 0.9	15.1 ± 1.6
C38	9.5 ± 2.0	8.0 ± 1.4	8.3 ± 1.6
C40	14.6 ± 1.9	13.8 ± 1.0	15.4 ± 1.8
C42	28.9 ± 4.4[b,c]	35.2 ± 4.3[c]	42.0 ± 4.6[b]
C34/C42§	1.10 ± 4.2[a]	0.78 ± 0.20	0.51 ± 0.13[a]

*Results of mean ± SD of 8 samples of cord blood.
†Carbon number represents the total number of sphingoshine and fatty acyl carbon atoms.
‡Values for a carbon number in different lipoproteins are significantly different when labelled: a, $p < 0.01$; b, $p < 0.001$; c, $p < 0.02$.
§Ratio of C34 to C42.

each lipoprotein fraction but was similar to that noted for lipoproteins in adult plasma (7). In LDL (Table 3) a higher proportion (30%) of short-chain SM (C34) than HDL_3 (21%) was found. By contrast, the long-chain (C42) SM were higher in HDL_3 (42%) than LDL (29%). The fatty acid composition of SM (Table 2) reflected these characteristics, primarily in the amounts of palmitic, behenic (24:0) and nervonic (24:1) fatty acids. Whereas palmitic acid was a major constituent in LDL, the HDL fraction was considerably richer in the long-chain fatty acids than LDL.

DISCUSSION

Detailed analyses of the molecular species of PC from lipoproteins of adult human serum have revealed a nearly equal distribution of the molecular species in VLDL, LDL and HDL, except for highly unsaturated species that are slightly higher in HDL_3 than in the other lipoproteins (9). However, a considerable difference in the distribution of SM molecular species are found among adult lipoproteins (7). In cord blood lipoproteins a differential distribution is observed for SM that is similar to the distribution seen in adult plasma.. Cord blood lipoproteins also had a marked difference in the distribution of the molecular species of PC. The cord blood LDL has much higher proportions of molecular species containing palmitic and C18 fatty acids than HDL. The

HDL was extremely rich in combinations of C18 and unsaturated C20 fatty acids. Thus, HDL from both adult (14) and cord blood has higher amounts of PC with polyunsaturated fatty acids than LDL, however the difference is more pronounced in cord blood. The fatty acid composition of cord blood PC agrees with previous studies, which demonstrated that C20 unsaturated fatty acids are present in higher concentrations than in adult serum (15).

The reasons for the pronounced unequal distribution of PL among cord blood lipoproteins are unknown at present. In adult serum, PC equilibration is enhanced by the PL exchange proteins present in the plasma (5,6). The PL exchange protein may possibly not be present in the fetus but appear sometime after birth. PL released from the catabolism of VLDL are also incorporated into HDL whereas LDL is generally considered to be a product of VLDL catabolism. Thus, if VLDL PL are precursors of the PL of HDL and LDL, the PL of the lipoproteins would be expected to be similar in composition unless specificity for phospholipid hydrolysis by lipolytic enzymes occurs. However VLDL is extremely low in cord plasma (16,17) and synthesis and turnover of VLDL is probably low because of the lack of extensive fat transport. Although the metabolic pathways for cord blood lipoproteins are essentially undefined, the present studies suggest that LDL and HDL_3 PL may arise from different metabolic pools. The lack of equilibration may be caused by a lack of PL exchange proteins (5,6) as well as low LCAT activity (8). Future studies on the changes in molecular species of cord blood PL in the neonate when dietary fat is consumed should provide clues to the effect of dietary lipids and fat transport on plasma PL and lipoprotein metabolism.

ACKNOWLEDGMENTS

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REFERENCES

1. Havel, R.J., Kane, J.P., and Kashyap, M.L. (1973) J. Clin. Invest. 52, 32-38.
2. Redgrave, T.G., and Small, D.M. (1979) J. Clin. Invest. 64, 162-171.
3. Redgrave, T.G. (1970) J. Clin. Invest. 49, 465-471.
4. Illingworth, D.R., and Portman, O.W. (1972) Biochim. Biophys. Acta 280, 281-289.
5. Ihm, J., Ellsworth, J., Chaitaing, B., and Harmony, J.A.K. (1982) J. Biol. Chem. 257, 4818-4827.
6. Tall, A.R., Forester, L.R., and Bongiovanni, G.L. (1983) J. Lipid Res. 24, 277-289.
7. Myher, J.J., Kuksis, A., Breckenridge, W.C., and Little, J.A. (1981) Can. J. Biochem. 59, 626-636.
8. Kuksis, A., Myher, J.J., Geher, K., Breckenridge, W.C., and Little, J.A. (1982) J. Chromatog. 230, 231-252.
9. Myher, J.J., Kuksis, A., Shepherd, J., Packard, C.J., Morrisett, J.D., Taunton, O.D., and Gotto, A.M. (1981) Biochim. Biophys. Acta 666, 110-119.
10. Lacko, A.G., Rutenberg, H.L., and Soloff, A. (1972) Lipids 7, 426-429.
11. Dolphin, P.J., Breckenridge, W.C., Dolphin, M.A., and Tan, M.H. (1983). Atherosclerosis (in press).
12. Folch, J., Lees, M., and Sloane Stanley, G.H. (1957) J. Biol. Chem. 226, 497-509.
13. Kuksis, A., Myher, J.J., Marai, L., and Geher, K. (1975) J. Chromatog. Sci. 13, 423-430.
14. Breckenridge, W.C., and Kuksis, A. (1968) J. Lipid Res. 9, 388-383.
15. Zee, P. (1967) Pediatrics 39, 82-87.
16. Winkler, L., Schlag, B., and Goetze, E. (1977) Clin. Chim. Acta 76, 187-191.
17. Dolphin, P.J., Breckenridge, W.C., Tan, M.H., Dolphin, M.A., Stewart, B., and Verpoorte, J. (1983) submitted for publication.

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Metabolism of Arachidonate in Rat Testis: Characterization of 26-30 Carbon Polyenoic Acids

W. McLEAN GROGAN, *Department of Biochemistry, Box 614 MCV Station, Medical College of Virginia, Virginia Commonwealth University, Richmond, VA 23298*

ABSTRACT

Fatty acid methyl esters of long-chain polyenoic fatty acids (LCPA) from rat testis injected with [1-^{14}C] arachidonate were analyzed and separated by reversed-phase high performance liquid chromatography (RP-HPLC). Earlier, all previously identified LCPA were prepared in high purity along with 4 previously unidentified fatty acids, which were further characterized by capillary gas chromatography (GC), catalytic hydrogenation and alkaline isomerization. Unidentified fatty acids proved to be 26:4, 26:5, 28:5 and 30:5. All of these LCPA incorporated ^{14}C from arachidonate (20:4) to specific activities that were comparable to that of 20:4 and previously identified metabolites of 20:4 and much greater than specific activities of 18:1n-9 or 22:6n-3. LCPA were analyzed on a capillary GC system capable of resolving known *cis-trans* and positional isomers of the n-3, n-6, n-7 and n-9 families of unsaturated fatty acids. Log plots of isothermal retention times and normal plots of temperature programmed retention times were linear (r = 0.999) in carbon number when values for known and previously unidentified LCPA of 4 or 5 double bonds, respectively, were coplotted. Thus, the newly identified fatty acids belong to the n-6 family of fatty acids synthesized from arachidonic acid.

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INTRODUCTION

Long-chain polyenoic acids (LCPA) with chain lengths in excess of 22 carbons have been reported in the testes of several species (1-4). The 24 carbon tetraene (24:4n-6) (by this convention, 24 carbons, 4 double bonds and 6 carbons from the methyl end to the highest numbered double bond (n-6), i.e., all *cis*-9, 12, 15, 18-tetracosatetraenoic acid) and pentaene (24:5n-6) are biosynthesized in the testis from arachidonic acid (20:4n-6) by a series of elongation and desaturation reactions. The presence of 26 carbon fatty acids has been suggested by gas chromatographic (GC) analysis of catalytically hydrogenated methyl esters of fatty acids extracted from rat testes (2). Incubation of mouse testes or intratesticular injection of mice with [^{14}C] arachidonate resulted in incorporation of ^{14}C into fatty acids with GC retention times greater than those of known 22 or 24 carbon polyenoic acids (5). Thus, circumstantial evidence that the testis biosynthesizes unsaturated fatty acids with chain lengths in excess of 24 carbons exists, but the specific fatty acids have not been isolated or identified. Because the number of potential isomers of LCPA having similar retention times becomes quite large as chain lengths increase, GC alone can provide only a tentative identification for a previously unknown LCPA (6). In the present study, we have used analytical and preparative reversed-phase high performance liquid chromatography (RP-HPLC) to confirm tentative identifications by capillary GC of 26, 28 and 30 carbon LCPA. We also provide evidence that these fatty acids are members of the n-6 family of LCPA, derived from arachidonic acid.

METHODS AND MATERIALS

Preparation of Fatty Acid Methyl Esters (FAME)

Total lipid was extracted with $CHCl_3$/MeOH (1:1, v/v) from decapsulated testes of adult male Sprague-Dawley rats (150-200 g; Flow Laboratories, Rockville, MD) by the procedure of Bridges and Coniglio (7). Extracts were reduced to dryness under vacuum on a rotary evaporator, redissolved in $CHCl_3$, dried under a stream of N_2 and transmethylated with $NaOCH_3$ by the procedure of Grogan et al. (4) to yield FAME. For studies of arachidonate metabolism, rats were anesthetized with ether and injected intratesticularly with 50 μCi/testis [1-^{14}C]-arachidonate (New England Nuclear, Boston, MA, 56 mCi/mmol) as the albumin complex. After 48 hr, the rats were sacrificed by cervical dislocation and testes were removed for preparation of FAME.

High Performance Liquid Chromatography (HPLC)

FAME (50-500 μg) were dissolved in 10-100 μl acetonitrile (AcCN) and separated on an HPLC system (Waters Assoc., Framingham, MA) consisting of 6000A pumps, U6K injector, 660 gradient programmer and a 7.8 mm x 30 cm reversed-phase column (μBondapak C_{18}). Effluent was monitored on a Schoeffel SF770 variable wavelength absorbance detector and

collector. FAME were eluted using a nonlinear gradient (no. 5 on the Waters 660) of 60-100% AcCN (solvent B) in 15% MeOH–H_2O (solvent A) for 1 hr at 2 ml/min. The gradient was begun 10 min after injection. A second protocol used to separate 18:2 from 22:5 consisted of a 66-100% gradient of MeOH in H_2O for 1 hr at 2ml/min. For measurement of radioactivity in eluent, fractions were collected at 0.5 min intervals into scintillation vials that were subsequently filled with scintillation fluid (Budget-Solve, Research Products International Corp.) for liquid scintillation counting. Counts were checked for quenching by the channels ratio method. For GC or chemical characterization, fractions were collected into 50 ml glass stoppered tubes, diluted 3-fold with H_2O and extracted 3× with 0.5 vol petroleum ether to recover FAME. FAME that had been previously characterized were identified by GC retention times. All solvents were HPLC grade. H_2O was 0.5 Mohm distilled H_2O redistilled in glass.

Gas Chromatography

Capillary GC of FAME was carried out on a Packard 427 GC equipped with flame ionization detector (FID), a splitless injector and a 0.75 mm × 60 m fused silica capillary column coated with 0.2 μm SP2340 (Supelco, Inc., Bellefont, PA). Argon carrier gas was used at a linear flow rate of 20 cm/sec. Column efficiency was in excess of 1,200 plates/m, sufficient for baseline resolution of the n-3 and n-6 isomers of 22:5. For temperature programmed analysis, a linear gradient from 120-260 C at 2 C/min was used with an initial time of 10 min. FAME were identified by comparing of retention times with those of commercial standards and biological samples of previously determined composition (5).

Chemical Characterization of LCPA

Aliquots of unsaturated FAME purified to 99% by preparative HPLC were catalytically hydrogenated by the method of Farquhar et al. (8) to yield corresponding saturated methyl esters. These were subsequently indentified by isothermal (180 C) GC as described above. Double-bond content of purified LCPA was determined by spectrophotometric analysis of alkaline isomerized aliquots. Petroleum ether extracts were dried under N_2, dissolved in 10% KOH-glycerol and heated to 180 C for 45 min as described in detail by Holman and Hays (9). Isomerized samples were analyzed for maximum wavelength of absorbance (λmax) on a Cary 210 recording spectrophotometer (Varian Assoc., Palo Alto, CA) scanning 220-400 nm. Unknowns were assigned double-bond values on the basis of previously reported λmax values (9,10) and by comparing them with known LCPA simultaneously subjected to the same analysis.

RESULTS

Total lipid extract from rat testis was transesterified to yield methyl esters of component fatty acids (FAME) and analyzed by RP-HPLC. Figure 1A shows the elution profile obtained by monitoring effluent for absorbance at 215 nm. All major unsaturated fatty acids were resolved by a single pass on this system with the exception of 18:2 and 22:5, which overlapped completely in retention time. Rechromatography, using the methanol-water gradient, completely resolved these fatty acids (relative retention times were 1.00 and 1.03, respectively), yielding 22:5 with no detectable impurities by HPLC or GC (data not shown). Only unsaturated FAME were detected by this method because detector response was roughly proportional to degree of unsaturation, although not in a linear mode (Table 1). This is best illustrated by a comparison of the percentage of area under the absorbance curve obtained for 18:1 by HPLC with the actual percentage of mass obtained by GC. For each fatty acid, detector response was proportional to concentration up to 5 μg of fatty acid injected.

Figure 2 shows a chromatogram obtained by capillary GC of the same FAME sample. This system resolved the n-7 and n-9 isomers of 16:1, the *cis-trans* isomers of 18:1n-9 and the n-3 and n-6 isomers of 22:5, as well as achieving baseline resolution of all commonly occurring fatty acids. The differing modes of separation of HPLC and GC are apparent in the fact that retention times are inversely proportional to the degree of unsaturation on the hydrophobic HPLC column and proportional to the degree of unsaturation on the polar GC column, given the same number of carbons in the respective FAME (Table 1 and Fig. 3). Retention times are proportional to carbon number with both methods, although the principle of separation is based on hydrophobicity in the case of HPLC and boiling point (molecular weight) and polarity in the case of GC. With the use of programmed decreases in solvent polarity (HPLC) or increases in column temperature (GC), the relationship between carbon number and retention time was linear (Correlation coefficient, $r>0.999$, in every case) for each homologous series (4 or 5 double bonds, respectively) allowing tentative identification of unknown FAME.

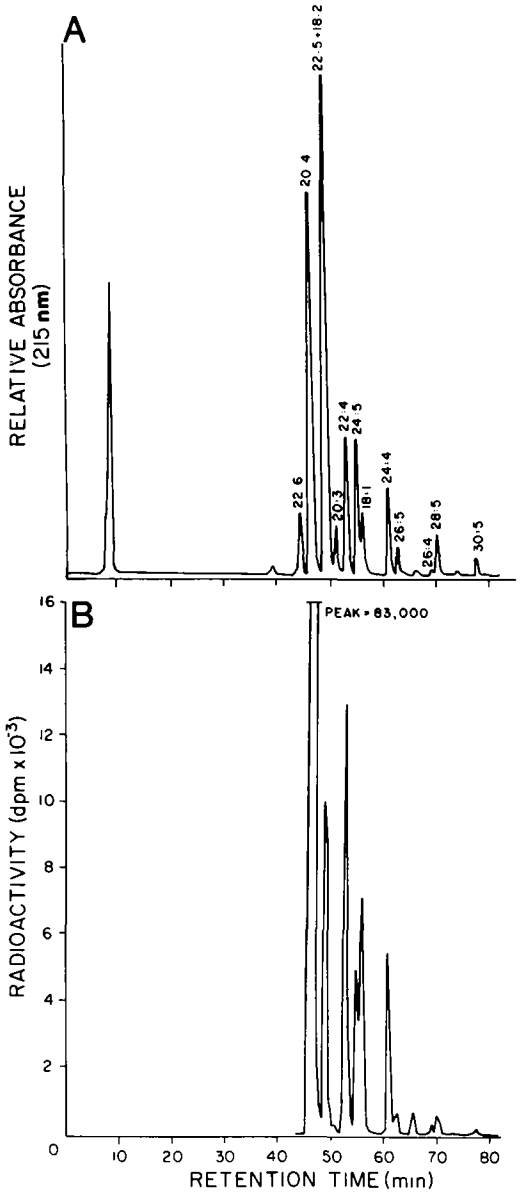

FIG. 1. RP-HPLC elution profile of unsaturated fatty acid methyl esters from rat-testis lipids. FAME were prepared by transesterification of total lipid extracts from rats injected intratesticularly with [1-^{14}C] arachidonate. FAME were eluted by a 1 hr nonlinear gradient of AcCN in 15% MeOH-H_2O as described in Methods. (A) Absorbance of eluent monitored at 215 nm. (B) Radioactivity of fractions collected at 0.5 min intervals.

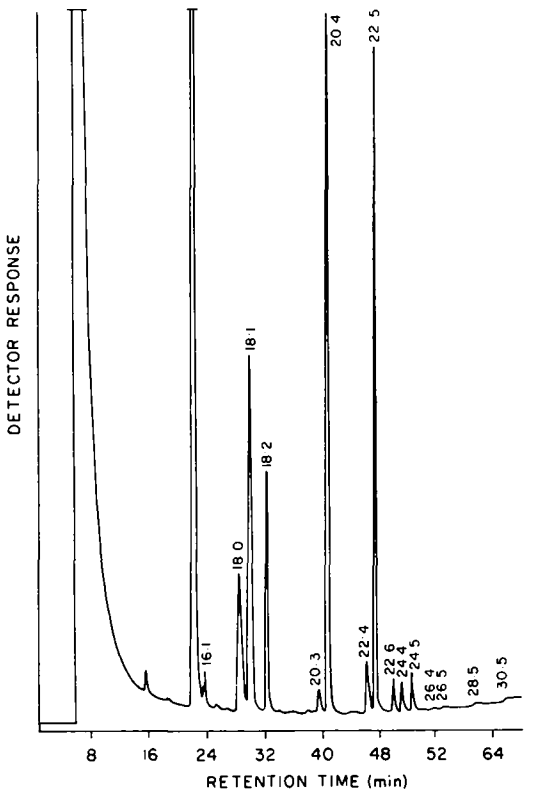

FIG. 2. Capillary GC of FAME from rat-testis lipids. FAME were prepared by transesterification of total lipid extracts and eluted by a linear temperature program from 120-260 C. Detector response is proportional to mass.

Identification of Unknown Fatty Acids

Unsaturated FAME coeluting with each major HPLC peak were collected and analyzed by capillary GC. Their relative retention times are given in Table 1. Most FAME could be identified by comparing retention times with those of standards or previously identified FAME from rat or mouse testes. Standards were unavailable for >24 carbon unsaturated fatty acids. Aliquots of unknown FAME were hydrogenated to corresponding 26, 28 and 30 carbon FAME and analyzed by GC. Aliquots of FAME were also isomerized by alkali to determine spectrophotometrically the number of double bonds present. As shown in Table 2, all 4 unknown FAME were isomerized to compounds absorbing with λmax of 317 or 348 nm, indicating 4 or 5 double bonds, respectively. Characteristic λmax were also obtained for other FAME prepared by HPLC, consistent with results reported by previous investigators using different preparative techniques and preparations of lesser purity (10,11).

Purified FAME were analyzed under isothermal conditions by a capillary GC system capable of resolving n-6 from n-3 and n-9 fatty acids. As shown in Figure 3, log plots of reten-

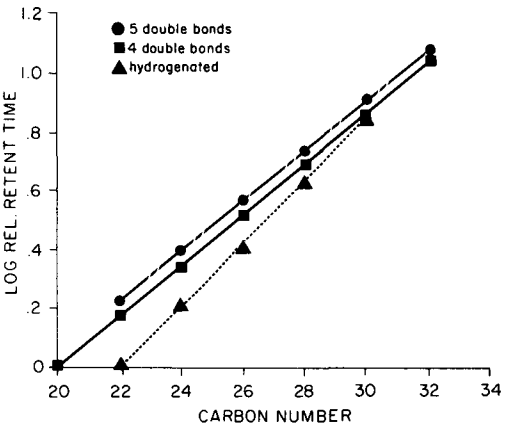

FIG. 3. Linear plots of isothermal GC log relative retention times vs carbon number for methyl esters of 20-30 carbon LCPA isolated by preparative HPLC. Retention times are relative to 20:4 for 4 and 5 double-bond LCPA and relative to 20:0 for hydrogenated samples. Points plotted beyond 30 carbons represent peaks detected by GC, but not further characterized. Correlation coefficient, $r \geq 0.999$ in each case.

TABLE 1

A Comparison of Capillary GC and HPLC Analyses of LCPA

Fatty acid	GC[a] Rel. ret. time	GC[a] Percentage of mass	HPLC[b] Rel. ret. time	HPLC[b] Percentage of mass
18:1n-9	0.70	22	1.23	1.2
18:2n-6	0.76	7.8	1.07	–
20:3n-6	0.97	1.6	1.11	1.5
20:4n-6	1.00	29	1.00	27
22:4n-6	1.16	2.9	1.16	4.0
22:5n-6	1.19	30	1.07	50[c]
22:6n-3	1.26	1.4	0.96	1.1
24:4n-6	1.29	1.7	1.33	2.6
24:5n-6	1.33	2.1	1.21	3.6
26:4n-6	1.42	0.2	1.52	0.2
26:5n-6	1.46	0.3	1.37	0.6
28:5n-6	1.58	0.3	1.54	0.8
30:5n-6	1.70	0.4	1.71	0.3

[a]Temperature programmed 120-260 C at 2 C/min. Retention times are relative to 20:4. FID response is linear with respect to mass. Percentage calculation includes only unsaturated fatty acids.

[b]Solvent programmed 60-100% AcCN in 15% MeOH-H_2O. Retention times are relative to 20:4. Absorbance at 215 nm is dependent on degree of unsaturation.

[c]Includes 18:2.

tion times for FAME of known n-6 fatty acids of 4 and 5 double bonds and those newly identified as 26:4, 26:5, 28:5 and 30:5 were remarkably linear in carbon number. Peaks were also seen having retention times appropriate for 32 carbon LCPA of 4 and 5 double bonds, although these were not further characterized.

TABLE 2

Characteristics of Previously Identified and Unknown LCPA Isolated by HPLC[a]

Fatty acid	Previously identified[b]	H_2 FA	λmax (nm)
20:3	x	20:0	279
22:4	x	22:0	317
22:5	x	22:0	348
22:6	x	22:0	374
24:4	x	24:0	317
24:5	x	24:0	348
26:4		26:0	317
26:5		26:0	348
28:5		28:0	348
30:5		30:0	348

[a]H_2 FA = fatty acid identified by GC after catalytic hydrogenation; λmax = maximum wavelength of absorbance following alkaline isomerization of double bonds.

[b]See references 1, 10, 11.

Biosynthesis of LCPA from Arachidonic Acid

Figure 1B shows the distribution of radioactivity in fractions collected during HPLC separation of FAME prepared from rat testis lipids 48 hr after intratesticular injection of [1-^{14}C]arachidonate. Radioactivity was incorporated into 22:4, 22:5, 24:4 and 24:5, which are known to be synthesized from 20:4 in the rat (2), and into 26:4, 26:5, 28:5 and 30:5, the newly identified LCPA. With the exceptions of 22:5 and 30:5, specific radioactivities of LCPA, which incorporated ^{14}C, were quite similar to the specific radioactivity of 20:4 from which the label was derived (Table 3). Specific radioactivities of all LCPA derived from 20:4 were 2- to 15-fold higher than that of 18:1, which was labeled during de novo synthesis from acetate derived from β-oxidation of 20:4 or a metabolite. The 22:6, wich is the predominant member of the n-3 family of LCPA, contained no detectable ^{14}C. The 22:5, which constituted 30% of the unsaturated fatty acid (Table 1), contained only 11% of total ^{14}C, but accounted for a corresponding 28% of ^{14}C in fatty acid metabolites of 20:4 (Table 3). In contrast, 22:4 constituted only 3% of unsaturated fatty acid but contained 10% of total ^{14}C and 25% of ^{14}C in metabolites of 20:4. Thus, the specific radioactivity of 22:4, which was typical of specific radioactivities of 20:4 and the 24-28 carbon metabolites, was 7-fold higher than that of 22:5.

DISCUSSION

The presence of 26 carbon fatty acids in testis has been suggested by GC analysis of hydrogenated fatty acid methyl esters prepared from rat-testis lipids (2). Presence of ^{14}C in FAME having GC retention times greater than those of known LCPA following intratesticular injection of mice or incubation of mouse testicular cells with [^{14}C]arachidonate suggested the biosynthesis of metabolites of arachidonate with chain lengths greater than 24 (5). In the present work, we have isolated and characterized 4 of these metabolites from rat testis, along with previously identified LCPA, and presented evidence that they are also derived from arachidonate.

LCPA of the 4 double-bond series have maximum wavelengths of absorbance near 317 nm following alkaline isomerization, characteristic of 4 double bonds (9-11), and yield saturated fatty acids of 20-26 carbons following catalytic hydrogenation. LCPA of the 5 double-bond series have maximum wavelengths of absorbance near 348 nm following alkaline isomerization, characteristic of 5 double bonds, and yield saturated fatty acids of 22-30 carbons folowing hydrogenation. The newly identified fatty acids are 26:4, 26:5, 28:5 and 30:5. Identifications of these LCPA are further corroborated by linear plots of GC (isothermal and programmed modes) and HPLC retention times vs carbon number in each homologous series. Because the capillary GC column used was able to resolve completely the n-6 and n-3 isomers of 22:5, the newly identified LCPA are almost certainly of the n-6 family derived from linoleate by way of arachidonate. This assignment is further supported by the incorporation of ^{14}C from arachidonate into each of the newly identified LCPA at specific radioactivities similar to those of 20:4, 22:4, 24:4 and 24:5 n-6 and much higher than the specific radioactivities of 18:1 or 22:6, which would only become labeled by de novo synthesis from acetate resulting from degradation of 20:4 or its metabolites. The major n-3 LCPA, 22:6, incorporated no detectable radioactivity.

RP-HPLC proved to be a highly efficient method for both analysis and preparation of each of the newly identified LCPA as well as those identified by previous investigators. Whereas conventional chemical techniques were used to support the identification of previously unknown LCPA, information derived from preparative HPLC and capillary GC is sufficient for unambiguous identification of the unknown components. A single pass on the HPLC was sufficient to purify most LCPA to 99% homogeneity. In cases where a partial or complete overlap of LCPA with another component took space (e.g., 22:5 and 18:2), rechromatography of the collected fraction with the alternate solvent system was sufficient to achieve complete homogeneity as judged by GC analysis.

TABLE 3

Relative Specific Radioactivities of Metabolites of [1-^{14}C] Arachidonate 48 hr After Intratesticular Injection

Fatty acid	Percentage of total radioactivity	Relative specific radioactivity
18:1	6	0.1
20:3	1	0.3
20:4	63	1.0
22:4	10	1.5
22:5	11	0.2
22:6	0	0
24:4	5	1.3
24:5	4	0.9
26:4	0.4	0.9
26:5	1	1.5
28:5	1	1.5
30:5	0.3	0.4

[a]Rats were injected intratesticularly with [1-^{14}C]-arachidonate. After 48 hr, lipids were extracted and transesterified to methanol. Methyl esters were isolated by HPLC and assayed for radioactivity by scintillation counter. Relative specific radioactivity = percentage radioactivity ÷ percentage mass, relative to 20:4.

The range of elongation of n-6 LCPA by rat testis is now at least 30 carbons and minor mass peaks from GC suggest the presence of LCPA with even greater chain lengths. These fatty acids of greater than 24 carbons comprise 1% or more of total fatty acid in the testis and are synthesized from arachidonate at rates that are sufficient to result in 48 hr equilibration of ^{14}C label between most of the precursor-intermediate-product LCPA pools involved. The specific radioactivity of 22:5 suggests a pool slowly equilibrating, probably because of the size and stability of the 22:5 pool. Accumulation of ^{14}C in 22:4, 24:4 and 26:4 suggests that the $\Delta 4$ desaturase, which catalyzes the reaction 22:4 → 22:5, is relatively slower than the elongation activities. The 24-30 carbon 5 double-bond homologs are probably also derived by sequential elongations from 22:5, since the $\Delta 6$, $\Delta 10$ and $\Delta 12$ desaturases required for biosynthesis from the corresponding 4 double-bond analogs are nowhere else in evidence. A $\Delta 8$ desaturase has been shown to participate in an alternate pathway for biosynthesis of arachidonate in rat testis (12). However, this desaturase probably would not act only on 20 and 26 carbon fatty acids.

Lack of accumulation of the 24-30 carbon LCPA in the testis despite rapid equilibration of ^{14}C among the 20-30 carbon LCPA suggests a relatively high rate of turnover of these LCPA, caused either by oxidation or retroconversion to shorter-chain analogs, as has been demonstrated for 22 carbon LCPA by several investigators (13-16). Unlike 22:5, the 24-30 carbon LCPA are probably not present in quantities sufficient to make them important storage forms of 20:4. However, as has been recently reported for 22:6n-3 (17), these LCPA may modulate biosynthesis of biologically active metabolites of the 20 carbon fatty acids or even give rise to biologically active metabolites themselves.

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REFERENCES

1. Bridges, R.B., and Coniglio, J.G. (1970) J. Biol. Chem. 245, 46-49.
2. Coniglio, J.G., Whorton, A.R., and Beckman, J.K. (1977) in Function and Biosynthesis of Lipids (Bazan, N.G., Brenner, R.R., and Giusto, N.M., eds.) pp. 575-589, Plenum Publishing Co., New York.
3. Holman, R.T., and Hofstetter, H.H. (1965) J. Am. Oil Chem. Soc. 42, 540-544.
4. Grogan, W.M., Farnham, W.F., and Szopiak, B.A. (1981) Lipids 16, 401-410.
5. Grogan, W.M., and Huth, E.G. (1983) Lipids 18, 275-284.
6. Hofstetter, H.H., Sen, N., and Holman, R.T. (1965) J. Am. Oil Chem. Soc. 42, 537-540.
7. Bridges, R.B., and Coniglio, J.G. (1970) Lipids 5, 628-635.
8. Farquhar, J.W., Insull, W., Jr., Rosen, P., Stoffel, W., and Ahrens, E.H., Jr. (1959) Nutr. Rev. 17 (suppl.), 1-29.
9. Holman, R.T., and Hayes, H. (1957) Methods Biochem. Anal. 4, 99-138.
10. Grogan, W.M., Coniglio, J.G., and Rhamy, R.K. (1973) Lipids 8, 480-482.
11. Davis, J.T., Bridges, R.B., and Coniglio, J.G. (1966) Biochem. J. 98, 342-346.
12. Albert, D.H., and Coniglio, J.G. (1977) Biochim. Biophys. Acta 489, 390-396.
13. Verdino, B., Blank, M.L., Privett, O.S., and Lundberg, W.O. (1964) J. Nutr. 83, 234-238.
14. Schlenk, H., Sand, D.M., and Gellerman, J.L. (1969) Biochim. Biophys. Acta 187, 201-207.
15. Sprecher, H. (1967) Biochim. Biophys. Acta 144, 296-304.
16. Bridges, R.B., and Coniglio, J.G. (1970) Biochim. Biophys. Acta 218, 29-25.
17. Aveldano, M.I., and Sprecher, H. (1983) J. Biol. Chem. 258, 9339-9343.

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Partial Purification of a Triacylglycerol Lipase Isolated from Steelhead Trout *(Salmo gairdneri)* Adipose Tissue

MARK A. SHERIDAN[a] * and WILLIAM V. ALLEN,[b] [a]*Department of Zoology and Cancer Research Laboratory, University of California, Berkeley, CA 94720 and* [b]*Department of Biological Sciences, Humboldt State University, Arcata, CA 95521*

ABSTRACT

A triacylglycerol (TG) lipase (EC 3.1.1.2), assayed by monitoring [1-^{14}C]-oleic acid release from [carboxyl-^{14}C]-triolein after liquid-liquid partition of the fatty acid from the unhydrolyzed triacylglycerol substrate, was isolated and partially purified from steelhead trout adipose tissue. The TG lipase was resolved from contaminating lipoprotein lipase by heparin-sepharose affinity chromatography and purified ca. 71-fold over the original fraction. Optimal enzyme activity occurred at pH 7.5. The purified enzyme migrated on SDS-polyacrylamide gels with an apparent molecular weight of 48,000.

Lipids 19: 347-352, 1984.

INTRODUCTION

The hydrolysis of depot fat in the rat (1-6) and the chicken (7) is catalyzed by hormone-sensitive triacylglycerol (TG) lipase, diacylglycerol (DG) hydrolase, monoacylglycerol (MG) hydrolase and cholesteryl ester (CE) hydrolase. The lipolytic action of these enzymes results in the mobilization of nonesterified fatty acids (8,9) and the subsequent transport of these acyl acids to peripheral tissues (10). The hormonal activation of the mammalian TG lipase (EC 3.1.1.2) has been well demonstrated (11-13). Purified hormone-sensitive TG lipase from both the chicken (7,14) and the rat (6,15) has been shown to be activated by a cAMP-dependent protein kinase.

Apart from the hydrolosis of long-chain glycerides in the dark muscle of rainbow trout observed by Bilinski and Lau (16), relatively little attention has been given to the study of lipid mobilization in fish or poikilotherms in general. Reported here is the partial purification of a triacylglycerol lipase isolated from the adipose tissue of the teleost fish, *Salmo gairdneri.*

MATERIALS AND METHODS

Experimental Animals

Juvenile steelhead trout, *Salmo gairdneri* (ca. 15 months of age), from a true-breeding anadromous population, were obtained from the California Department of Fish and Game Iron Gate Fish Hatchery. The fish were transferred to holding tanks (14 C) in the laboratory and left undisturbed for 96 hr. Animals were sacrificed by rapid transection. Mesenteric fat attached to the posterior portion of the stomach and along the dorsal and ventral surfaces of the intestine were removed and stored at −120 C until used.

Enzyme Purification

Adipose tissue from 5-10 individuals was homogenized in 2 vol cold (4 C) Buffer A (0.25 M sucrose, 25 mM Tris-HCl, 1.0 mM EDTA, pH 7.4) and the homogenate centrifuged at 1,000 *g* for 10 min at 0 C. The resulting overlying fat cake was removed and the infranatant (Infranatant I) centrifuged at 110,000 *g* for 1 hr at 0 C. A 0.2 M acetic-acid solution was added by drops to the supernatant fraction until the pH reached 5.2. After 20 min, the precipitate (pH 5.2 precipitate) was collected by centrifugation at 3,000 *g* for 15 min at 0 C. The pH 5.2 precipitate was resuspended in Buffer B [25 mM Tris-HCl, 1.0 mM EDTA, pH 7.4, 4 C] to 1/10 the vol of Infranatant I. NaCl was added to the pH 5.2 fraction to yield a final salt concentration of 0.5 M, then centrifuged at 10,000 *g* for 15 min at 0 C. The resulting infranatant (Infranatant II) was loaded on a heparin-sepharose affinity column (1.5 × 20 cm) previously equilibrated with Buffer C [0.5 M NaCl, 10 mM Tris-HCl, pH 7.4] at 4 C (7). The flow rate was adjusted to 35 ml/hr and 1.5 ml fractions were collected. After 100 ml of effluent, the eluent was changed to Buffer D [1.5 M NaCl, 10 mM Tris-HCl, pH 7.4] in order to elute lipoprotein lipase (LPL). The column effluent was continuously monitored at 280 nm with a Beckman model 24 Spectrophotometer.

The heparin-sepharose affinity column was prepared by coupling bovine lung heparin (sodium salt, Sigma Chemical Co., St. Louis,

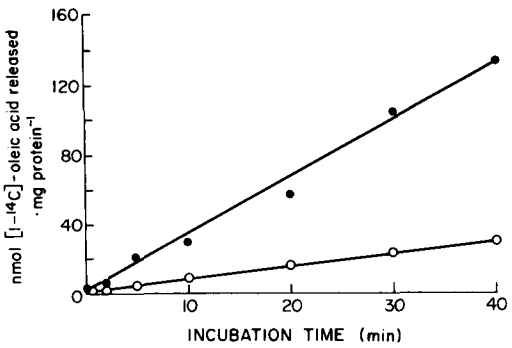

FIG. 1. Time course of [1-^{14}C]-oleic acid hydrolysis from [carboxyl-^{14}C]-triolein incubated with (•) and without (○) pH 5.2 enzyme preparation (ca. 2 mg protein) at 25 C. The substrate mixture contained 0.12 μmol triolein, 45 nCi [carboxyl-^{14}C]-triolein in a Tris-Maleate buffer, pH 7.5, with 2.8% (w/v) bovine serum albumin. Results expressed as the mean of 3 experiments.

MO) to CNBr-activated sepharose 6MB (Sigma) by means of a modification of the method suggested by Pharmacia (17). The heparin was allowed to incubate with the sepharose beads overnight at 4 C in a shaker bath. Excess CNBr groups were blocked with 0.2M glycine, pH 8.0. The gel was stored in Buffer C at 4 C between runs.

Enzyme Assay

Triacylglycerol (TG) lipase activity was assayed by a modification of the method of Khoo and Steinberg (18). A stock substrate mixture of 12 μmol triolein (Sigma) and 4.5 μCi [carboxy-^{14}C]-triolein (New England Nuclear, Boston, MA; specific activity 99.7 mCi/m mol) was dissolved in absolute ethanol, made up to a final volume of 1.0 ml and stored in the dark at 4 C until used. A working substrate solution was prepared by mixing 4.9 ml H_2O, 2.0 ml 0.2 M Tris-Maleate buffer with 10% (w/v) bovine serum albumin (Sigma), pH 7.5, and 0.1 ml stock substrate solution. For the determination of pH optima, 0.2 M Tris-Maleate (5.5 - 8.5) buffer or 0.1 M citrate-phosphate (4.5 - 5.0) buffer was employed. For TG lipase assays, 0.7 ml of working substrate solution was placed in a disposable glass culture tube and the incubation initiated by the addition of 0.1 ml enzyme solution. Just after the addition of enzyme, a 50 μl aliquot was removed for [1-^{14}C]-oleic acid specific radioactivity estimation. The incubation was stopped by the addition of 3.0 ml of fatty acid extraction solvent (chloroform/methanol/benzene, 1:2.4:2, v/v/v). The mixture was brought to pH 11.5 with 0.1 M NaOH and mixed thoroughly. Separation into 2 phases was hastened by centrifugation at 1,000 *g* for 10 min at room temperature. A 1.5 ml aliquot of the upper aqueous phase was assayed for radioactivity in a Packard Model 3320 liquid scintillation counter mixed with Aquasol-2 (New England Nuclear). Quenching was corrected by the channels ratio method. Units of enzyme activity, expressed as nmol [1-^{14}C]-oleic acid released/hr, were calculated after correcting for autolytic hydrolysis (estimated from duplicate assay tubes not containing enzyme solution). Specific enzyme activity is expressed as units/mg protein.

Lipoprotein lipase activity was assayed in a similar manner, except that the working substrate was prepared from 4.8 ml H_2O, 2.0 ml Tris-Maleate buffer, pH 8.5, 0.1 ml trout serum and 0.1 ml stock substrate solution.

Electrophoresis

SDS-polyacrylamide disc gel electrophoresis (10% [w/v] monomere concentration) was carried out using the method of Weber and Osborn (19). Standards and samples were boiled in 1% (w/v) SDS for 5 min (i.e., non-reducing). Gels were stained for protein with 0.025% (w/v) Coomassie blue in isopropanol/acetic acid (2.5:1, v/v). A standard mixture containing lysozyme, soybean trypsin inhibitor, carbonic anhydrase, ovalbumin, bovine serum albumin and phosphorylase B was obtained from Bio-Rad, Richmond, CA.

Proteins from Coomassie stained gel slices were eluted (24 hr at 4 C with gentle swirling) in 0.1 ml Buffer B after 1 hr preincubation in 0.25% (v/v) acetic acid.

Protein Determination

Protein was determined by the method of Lowry et al. (20) using bovine serum albumin (Sigma) as standard.

RESULTS

Preliminary experiments on the time course of [1-^{14}C]-oleic acid release and the effect of incubation temperature were carried out to determine optimal enzyme assay conditions. Hydrolysis of [1-^{14}C]-oleic acid from [carboxyl-^{14}C]-triolein incubated at 25 C was linear for up to 40 min (Fig. 1). Subsequent assays were performed for 30 min as longer incubation periods were judged to have undesirably high blanks (probably resulting from increased autolytic hydrolysis of substrate). The effect of incubation temperature on TG lipase activity in the pH 5.2 fraction appears in

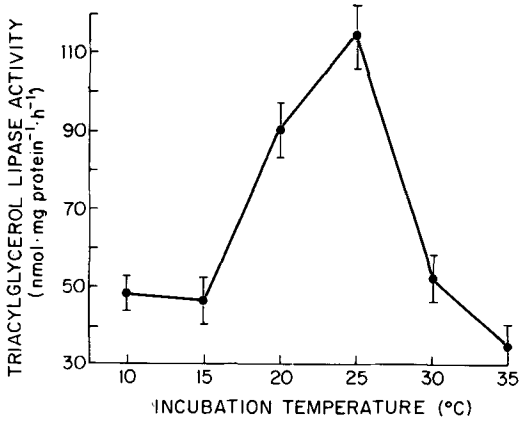

FIG. 2. Effect of assay temperature on triacylglycerol lipase activity in the pH 5.2 fraction. Aliquots of the pH 5.2 fraction were incubated in a Tris-Maleate buffer, pH 7.5, containing 2.8% (w/v) bovine serum albumin, 0.12 μmol triolein and 45 nCi [carboxyl-^{14}C]-triolein. Results presented as the mean ± SEM for 3 experiments.

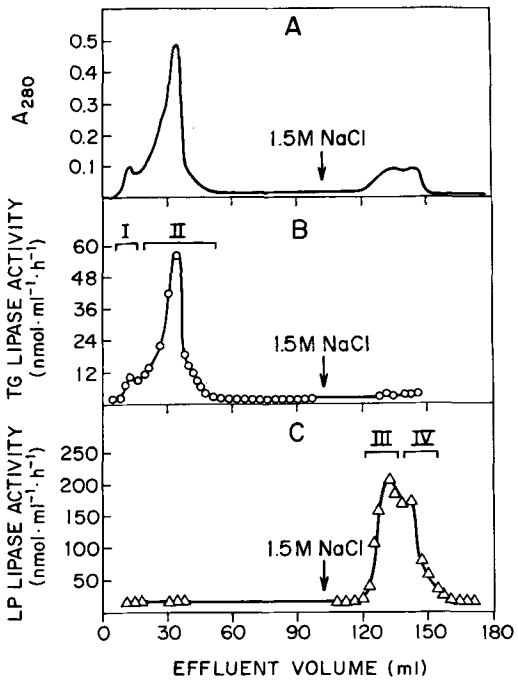

FIG. 3. Chromatography of the pH 5.2 fraction on a heparin-sepharose 6MB affinity column (1.5 × 20 cm). (A) A_{280}, (B) triacylglycerol lipase activity and (C) lipoprotein lipase activity. The column was eluted with a 10 mM Tris-HCl buffer solution containing 0.5 M NaCl, pH 7.4 (Buffer C). After 100 ml of effluent were collected, the eluent was changed to a Tris-HCl buffer solution containing 1.5 M NaCl (Buffer D). The flow rate was 35 ml/hr and 1.5 ml fractions were collected. Triacylglycerol lipase was assayed in a Tris-Maleate buffer, pH 7.5, containing 2.8% (w/v) bovine serum albumin, 0.12 μmol triolein and 45 nCi [carboxyl-^{14}C]-triolein at 25 C. Lipoprotein lipase activity was assayed in a similar manner except that the incubation media contained 14 μl of trout serum and the pH was adjusted to 8.5 (TG, triacylglycerol; LP, lipoprotein).

Figure 2. Maximal enzyme activity was obtained at 25 C; therefore, this temperature was used in subsequent assays.

Resolution of TG lipase from lipoprotein lipase was achieved by heparin-sepharose affinity chromatography of the resuspended pH 5.2 precipitate. The elution profile of total protein, TG lipase activity and LP lipase activity is shown in Figure 3. Most of the TG lipase activity in the pH 5.2 fraction emerged from the heparin-sepharose affinity column when eluted with Buffer C (containing 0.5 M NaCl) as a sharp, slightly retarded peak (Fraction II, Fig. 3B), but a small amount appeared just after the void (Fraction I, Fig. 3B). After 100 ml of effluent were collected, the eluent was changed to Buffer D (containing 1.5 M NaCl), thus eluting the LPL bound to the ligand of the affinity column. LPL activity was eluted in a broad, twin-peaked fraction (Fractions III and IV, Fig. 3C). Fractions I and II were assayed for LPL activity with no significant activity recorded (Fig. 3C), indicating little or no contamination with LPL. Similarly, Fractions III and IV were assayed for TG lipase with no appreciable activity noted (Fig. 3B), suggesting that the majority of the TG lipase was eluted in Fractions I and II.

The effect of pH on TG lipase activity is shown in Figure 4. Assays were performed in an appropriate buffer at the pH values indicated. For ease of comparison, activity is expressed in relation to optimal pH. The pH profile of Infranatant I (Fig. 4A), resulting from the original homogenate and containing both membrane and soluble enzymes, appears bimodal with optimal activity at pH 7.5 and enhanced hydrolase activity at pH 5.0. The effect of pH on the soluble enzyme pH 5.2 fraction (Fig. 4B) resulted in a monomodal profile with optimal activity at pH 7.5. The pH profiles of the Fraction I and Fraction II enzyme preparations appear in Figure 4B. Three successive pooled fractions (4.5 ml) of each peak (Fraction I and Fraction II) were assayed for TG lipase activity at each of the pH values indicated. The pH optimum for both preparations was 7.5.

The molecular weight of the TG lipase preparation was estimated by SDS-polyacrylamide

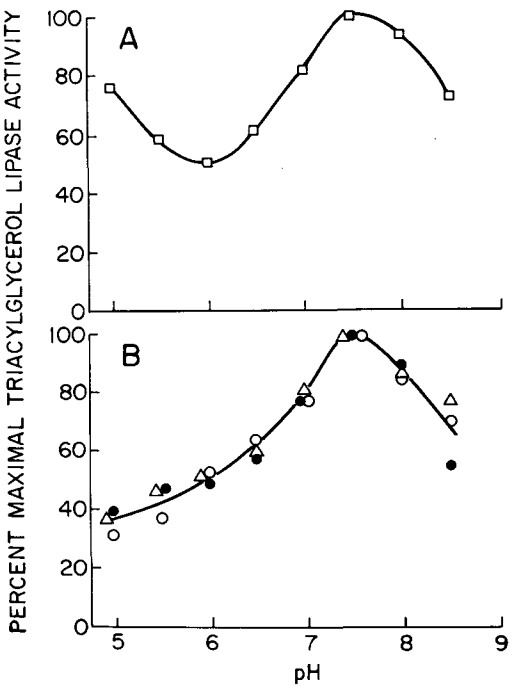

FIG. 4. Effect of pH on tricylglycerol lipase activity. (A) Infranatant I, (B) pH 5.2 fraction (○), heparin-sepharose affinity chromatography Fractions I (△) and II (●). Aliquots of the individual fractions were incubated at 25 C in either a citrate-phosphate or Tris-Maleate buffer containing 2.8% (w/v) bovine serum albumin, 0.12 μmol triolein and 45 nCi [carboxyl-^{14}C]-triolein at the pH values indicated. Activities are expressed in relation to optimal pH and represent the mean values of 3 experiments.

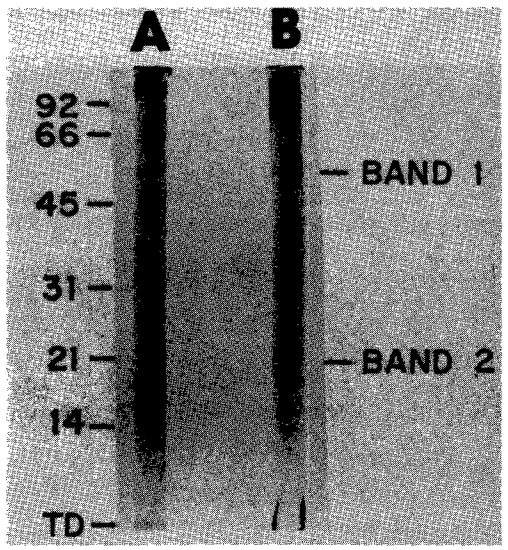

FIG. 5. SDS-polyacrylamide disc gel electrophoresis of heparin-sepharose affinity fractions. Gel A, aliquot from 3 successive pooled fractions (4.5 ml) in heparin-sepharose affinity chromatography Fraction I. Gel B, aliquot from 3 successive pooled fractions (4.5 ml) in heparin-sepharose affinity chromatography Fraction II. Band 1 M_r = 48,000 daltons; Band 2 M_r = 16,000 daltons. Gels were stained for protein with 0.25% (w/v) Coomassie blue.

gel electrophoresis. One major protein band M_r = 48,000, was observed in the heparin-sepharose affinity Fraction I (Fig. 5, gel A). Two protein bands, M_r = 48,000 and M_r = 16,000, were observed in heparin-sepharose affinity Fraction II (Fig. 5, gel B). In order to localize lipase activity in the Fraction II bands, each band was sliced from the gel and the proteins eluted in 0.1 ml Buffer B. The TG lipase activity determined on each eluate appears in Table 1. Seventy-eight percent of the total recovered activity was associated with the M_r = 48,000 protein (band 1, Fig. 5).

Triacylglycerol lipase activity was determined on aliquots (0.1 ml) removed from the original infranatant (Infranatant I), the 110,000 *g* supernatant, the pH 5.2 fraction and heparin-sepharose affinity chromatography Fraction II. A summary of the purification appears in Table 2. Most of the hydrolase activity in the fat-free infranatant (Infranatant I) was recovered in the soluble enzyme (110,000 *g* supernatant) fraction. The lipoprotein lipase free heparin-sepharose Fraction II was purified 71-fold over the original fraction with 37% recovery of the enzyme.

TABLE 1

Localization of Triacylglycerol Lipase Activity in the M_r = 48,000 Dalton and M_r = 16,000 Dalton Protein Bands from Heparin-Sepharose Affinity Chromatography Fraction II

Fraction	Protein[a] (mg)	Specific enzyme activity (units/mg protein)
Band 1	0.024	684
Band 2	0.018	181
Total	0.042	865
Recovery[b]		37%

[a]Protein content in bands was estimated by densitometry. Total protein equals amount of protein applied to electrophoresis gels.

[b]Recovery calculated as percentage of specific enzyme activity observed in heparin-sepharose affinity chromatography Fraction II.

DISCUSSION

The results of the present study indicate that

TABLE 2

Summary of the Purification of a Triacylglycerol Lipase from Steelhead Trout *(Salmo gairdneri)* Adipose Tissue[a,b]

Fraction	Total enzyme activity (units)[c]	Total protein (mg)	Specific enzyme activity (units/mg protein)	Yield (%)	Purification (fold)
Infranatant I	316	9.54	33	100	1
110,000 *g* supernatant	293	5.45	54	93	1.6
Resuspended pH 5.2	249	2.18	114	78	3.4
Heparin-sepharose Fraction II	117	0.05	2340	37	71

[a]Data presented are the mean values of 3 experiments.

[b]Ca. 5.0 g of adipose tissue from 5-10 animals were used as starting material.

[c]Units = nmol ^{14}C-oleic acid released · h^{-1} at 25 C.

steelhead trout adipose tissue contains a neutral triacylglycerol lipase enzyme associated with the soluble cytosolic fraction. This conclusion is supported by the observation that most of the hydrolase activity in the original fat-free infranatant (Infranatant I) was recovered in the 110,000 *g* supernatant (Table 2) and that no acid lipase activity was observed in the 110,000 *g* supernatant or subsequent fractions (Fig. 4B).

The pH optimum for each of the purified adipose tissue fractions (Infranatant I, pH 5.2, heparin-sepharose fraction I and heparin-sepharose fraction II) was ca. pH 7.5. This value is the same as that reported for trout lateral line muscle triglyceride hydrolase activity by Bilinski and Lau (16). Enhanced hydrolase activity at pH 5.0 in the Infranatant I fraction appears to be associated with membrane elements. This observation is consistent with previous reports of acid lipase activity (21-24).

The apparent molecular weight of trout adipose tissue lipase is 48,000. This is indicated by the presence of a single protein band of M_r = 48,000 in the early heparin-sepharose affinity fraction (fraction I), and the localization of triacylglycerol lipase activity from the M_r = 48,000 band in the partially included heparin-sepharose affinity fraction (fraction II). In the few species in which purified lipase preparations have been obtained, a wide range of molecular weight proteins have been identified. In the rat, an M_r = 85,000 protein has been identified by a number of investigators (6,15, 25). Bergland et al. (26) isolated a M_r = 42,000 protein from chicken adipose tissue that was fully capable of being phosphorylated when incubated with cAMP-dependent protein kinase and ATP-Mg.

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REFERENCES

1. Rizack, M.A. (1961) J. Biol. Chem. 236, 657-662.
2. Vaughan, M., Berger, J.E., and Steinberg, D. (1964) J. Biol. Chem. 239, 401-409.
3. Huttunen, J.K., and Steinberg, D. (1971) Biochim. Biophys. Acta 239, 411-427.
4. Arnaud, J., and Boyer, J. (1974) Biochim. Biophys. Acta 337, 165-168.
5. Pittman, R.C., Khoo, J.C., and Steinberg, D. (1975) J. Biol. Chem. 250, 4504-4511.
6. Fredrikson, G., Stralfors, P., Nilsson, N.O., and Belfrage, P. (1981) J. Biol. Chem. 256, 6311-6320.
7. Khoo, J.C., Steinberg, D., Huang, J.J., and Vagelos, P.R. (1976) J. Biol. Chem. 251, 2882-2890.
8. Vaughan, M., and Steinberg, D. (1965) in Handbook of Physiology (Renold, A.E. and Cahill, G.F., eds.) Section 5, pp. 239-251, Williams and Wilkins, Baltimore, MD.
9. Fredrickson, D.S., and Gordon, R.S. (1958) Physiol. Rev. 38, 585-630.
10. Allen, W.V. (1976) Amer. Zool. 16, 631-647.
11. Hollenberg, C.H., Raben, M.S., and Astwood, E.G. (1961) Endocrinology 68, 589-598.
12. Björntorp, P., and Furman, R.H. (1962) Amer. J. Physiol. 203, 316-322.
13. Vaughan, M., and Steinberg, D. (1963) J. Lipid Res. 4, 193-199.
14. Khoo, J.C., and Steinberg, D. (1974) J. Lipid Res. 15, 602-610.
15. Belfrage, P., Jergil, B., Stralfors, P., and Tornqvist, H. (1977) FEBS Lett. 75, 259-264.

16. Bilinski, E., and Lau, Y.C. (1969) J. Fish. Res. Bd. Can. 26, 1837-1866.
17. Pharmacia Technical Bulletin (1979) Affinity Chromatography: Principles and Methods, Pharmacia Fine Chemicals, Uppsala, Sweden.
18. Khoo, J.C., and Steinberg, D. (1981) in Methods in Enzymology (Lowenstein, J.M., ed.) Vol. 71, pp. 627-636, Academic Press, New York.
19. Weber, K., and Osborn, M. (1969) J. Biol. Chem. 244, 4406-4412.
20. Lowry, O.H., Rosenberg, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
21. Mahadevan, S., and Tappel, A.L. (1968) J. Biol. Chem. 243, 2849-2854.
22. Guder, W., Weiss, L., and Weiland, O. (1969) Biochim. Biophys. Acta 187, 173-185.
23. Loffler, G., and Weiss, L. (1970) in Adipose Tissue: Regulation and Metabolic Functions (Jeanrenaud, B. and Hepp, D., eds.), pp. 32-36, Academic Press, New York.
24. Bilinski, E., Jonas, R.E.E., and Lau, Y.C. (1971) J. Fish. Res. Bd. Can. 28, 1015-1018.
25. Khoo, J.C., Berglund, L., Jensen, D., and Steinberg, D. (1980) Biochim. Biophys. Acta 619, 440-444.
26. Berglund, L., Khoo, J.C., Jensen, D., and Steinberg, D. (1980) J. Biol. Chem. 255, 5420-5428.

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METHODS

HPLC of Plasmalogen-Containing Phosphatidylcholine Under Reverse-Phase or Argentation Conditions

WILLIAM N. MARMER,* THOMAS A. FOGLIA and **PETER D. VAIL,** *Agricultural Research Service, USDA, Eastern Regional Research Center, Philadelphia, PA 19118*

ABSTRACT

Two approaches to the high pressure liquid chromatography (HPLC) isolation of intact plasmalogens were investigated. The first used reversed-phase HPLC and sought to take advantage of subtle differences in the hydrophobicity of the alk-1-enyl chain from the acyl counterpart. On a C-18 column, bovine heart phosphatidylcholine (PC), which was 47% plasmalogen, was separated into a number of fractions that differed in their molecular species composition. One combination of fractions amounted to a 26% yield of PC enriched to 82% plasmalogen. The second approach sought to take advantage of the uniquely electron-rich functionality of the plasmalogens, the alk-1-enyl ether double bond, and its potential to coordinate with heavy metal ions. Specifically, bovine heart PC was applied to a cation-exchange type HPLC column in the silver ion mode. Although complete exchange of all the active sites of the column with silver ion led to complete retention of PC, partial activation with silver ion resulted in the separation of the PC into fractions, according to the degree of unsaturation. Plasmalogen-rich fractions eluted last and remained intact during the process. One combination of these fractions amounted to a 49% yield of PC enriched to 72% plasmalogen. Use of a cation-exchange system in the mercuric ion mode led to on-column hydrolysis of the plasmalogen; with palladium ion, the metallic species was stripped from the column by the eluting lipid.
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INTRODUCTION

Chromatographic separation of intact plasmalogens (alk-1-enyl phosphatides) has eluded researchers for years. The behavior of these species during high performance liquid chromatography (HPLC) has been ignored, mainly because HPLC studies have largely been confined to model diacylphosphatides or to natural phosphatides with little or no plasmalogen content (e.g., liver, egg yolk or soy phosphatidylcholine [PC]). The plasmalogen content of natural phosphatides from muscle or heart tissue is considerable, however. The phosphatide selected for the present investigation, bovine heart PC, is ca. half plasmalogen.

The alk-1-enyl group is a unique and highly electron-rich double bond, which is easily protonated and capable of complexing with electrophilic species (such as tetracyanoethylene [1], maleic anhydride [2] and mercuric ion [3]), to a degree unseen with isolated double bonds. With the exception of early argentation thin layer chromatography (TLC) work discussed below, however, such interactions have not been used to attempt the isolation of intact plasmalogen from nonplasmalogen analogs. Typically, the chromatographic behavior of plasmalogen is governed by the polar character of the phosphobase group that it has in common with its nonplasmalogen counterparts. Successful chromatographic separations, therefore, have been achieved by first removing the polar group by enzymatic hydrolysis (4) or chemical reduction (5), or by conversion of the polar group to a nonpolar derivative (6,7). The chromatography that ensues thus ceases to be a separation of intact plasmalogen. These types of chromatographic analyses have been reviewed (8).

Successful separation of molecular species of intact phosphatides has been achieved by argentation TLC. Fractionation was based on unsaturation content, despite the common polar group shared by all the species. Although most reports dealt with phosphatides of negligible plasmalogen content (9-13), 2 studies dealt with plasmalogen-rich bovine heart phosphatides–PC (14) and phosphatidylethanolamine (PE) (15). These studies demonstrated that the lipids could be fractionated into overlapping bands on Silica Gel G plates that had been predeveloped with saturated silver nitrate. The overall separations seemed to be a function of the total degree of unsaturation, though some of the bands were found to be enriched in plasmalogen.

*To whom correspondence should be addressed.

Argentation HPLC of phosphatides, on the other hand, has been virtually ignored. It has been used to supplement reversed-phase HPLC (RP-HPLC) to isolate molecular species of sphingomyelin (SM) (16), whereby the argentation technique enabled the separation of critical pairs unseparable on reversed-phase columns but differing in unsaturation content. Similarly separated were critical pairs of egg-yolk PC (17).

On a different tack, another group recently has reported on the successful separation of molecular species of (plasmalogen-free) rat-liver phosphatides by RP-HPLC (18). Under these conditions, too, the influence of the polar group is minimized, this time by the dominance of hydrophobic interactions.

The present research was done to investigate the behavior of plasmalogen-rich (47 mol %) bovine heart PC when subjected to argentation and other charge-transfer HPLC, and to RP-HPLC.

EXPERIMENTAL

HPLC Instrumentation

Chromatography was run on a Beckman Model 334 Gradient Liquid Chromatograph, consisting of a Model 210 sample injection valve, a Model 421 system controller and Model 110A pumps (Beckman Scientific Instruments Division, SmithKline-Beckman, Inc., Berkeley, CA), and supplemented by an LKB Model 238 Uvicord S UV detector (LKB Instruments, Inc., Gaithersburg, MD) and an MFE Model 2125B dual pen recorder (Allen Data Graph, Inc., Salem, NH). Monitoring of column effluent was done at 206 nm.

Two strong cation-exchange columns were used; initial runs were on a Nucleosil 5 SA column, 4.0 mm i.d. x 20 cm, 5 micron particle size (Macherey-Nagel GmbH, Düren, FRG and Rainin Instruments, Inc., Woburn, MA); later runs were on a Chromegabond P-SCX column, 4.6 mm i.d. x 30 cm, 10 micron particle size (ES Industries, Marlton, NJ). For RP-HPLC, an Ultrasphere ODS column was used, 4.6 mm i.d. x 25 cm, 5 micron particle size (Beckman).

Other Instrumentation

Gas chromatographic (GC) analysis of fatty acid methyl esters (FAME) and aldehydes was performed on a Hewlett-Packard Model 5880A level 4 flame ionization capillary gas chromatograph (Hewlett-Packard, Avondale, PA) equipped with a 100 m 0.25 mm i.d. SP 2340 glass column (Quadrex, New Haven, CT). Identification of FAME was aided by standards reported in an earlier publication (19). Additionally, structural identification of individual aldehydes and FAME was confirmed by GC-mass spectrometry (MS), using a Hewlett-Packard Model 5995 instrument equipped with a Hewlett-Packard 15 m OV-101 fused silica capillary column (Hewlett-Packard, Avondale, PA); the oven was programmed from 150 to 220 C at 8 C/min.

HPLC Conditions

Cation-exchange columns were treated with silver nitrate as follows: the column first was converted from the acid mode to the sodium mode by treatment with 0.1 N $NaNO_3$ until no further detection of eluted acid (pH paper) occurred. This was followed by flushing with doubly distilled water. Then, after switching to the solvent system to be used (aqueous methanol), 0.1 N aqueous $AgNO_3$ was introduced incrementally (by 10 μl injections) to establish optimum column load. After each incremental addition, the column was evaluated with the lipid solution (20 μl of bovine heart PC in chloroform, 10 mg/ml [Sigma Chemical Co., St. Louis, MO]). Column capacity was determined by charging the column with 0.1 N HNO_3, flushing with water, collecting the effluent during charging with 0.1 N $NaNO_3$ and finally titrating the released acid with 0.1 N NaOH. In a manner analogous to charging with silver ion, the same procedure was used to incorporate palladium II ion (0.1 M $PdCl_2$ in methanol), mercuric ion (using 0.1 M aqueous mercuric acetate) and phenylmercuric ion (using 0.1 M phenylmercuric acetate in 10% aqueous methanol). (Warning: handle toxic mercuric compounds with care.)

Run conditions were as follows (solvent A = water, B = methanol): Macherey-Nagel column in Ag mode-flow, 1 ml/min; solvent gradient = 100% B to 70% B over 15 min, then isocratic; ES column in Ag mode--flow, 2 ml/min through 8 min, then to 4 ml/min over 6 min, then constant flow at 4 ml/min; solvent = 85% B; Macherey-Nagel column in Hg mode–flow, 1 ml/min; solvent = 80% B; ES column in palladium mode–flow = 1 ml/min; solvent = 80 or 85% B; Beckman reversed-phase column–flow = 2.5 ml/min; solvent = B/A/acetonitrile 95:4:1 0.13 M in choline chloride (a modification of the method of Patton et al. [18]).

Highly unsaturated PC fractions that failed to elute from the charge-transfer columns by the solvents of choice were released by injection of 80 μl of 1-hexene at the end of each run, in accordance with published results on medium pressure liquid chromatography of neutral lipids (20,21).

Metal ion leakage from the columns was detected as follows: Ag–white precipitate with aqueous NaCl; Hg and Pd–purple color with s-diphenylcarbazone (Fisher, Fairlawn, NJ).

Analysis of Lipid Fractions

Lipid fractions were analyzed for plasmalogen content by acid cleavage of the plasmalogen to aldehyde and 2-acylglycerophosphocholine, TLC to separate the fragments from unreacted nonplasmalogen PC and elemental phosphorus analysis (22) of the 2 phosphorus-containing species, according to the Horrocks procedure (23).

Aldehydes that were released from plasmalogen by acid treatment were analyzed directly by GC or converted to FAME after a mild oxidation during a multistep TLC procedure as follows: a lipid spot at the origin of a Silica Gel G TLC plate (5 x 20 cm, 250 m, Analtech, Newark, DE) was first treated with HCl vapor (23). The plate then was developed 7 cm using hexane/ether (80:20, v/v). Phosphorus-containing species remained at the origin. The aldehyde spot, which was just below the solvent front and located by spraying a parallel spot with fuchsin/bisulfite, was sprayed with a saturated solution of ceric sulfate in 0.5 N H_2SO_4. The plate was warmed for a minute on a hot plate (at the lowest setting). The cooled plate was redeveloped 14 cm in the same solvent mixture to separate any unreacted aldehyde (high R_f) from the product fatty acid (which only moved slightly from the region sprayed with ceric sulfate). The fatty acid spot was visualized under UV light after spraying with 1,6-diphenylhexatriene. Redevelopment in hexane removed the visualization reagent but left the fatty acid at its former position. The fatty acid spot was removed by scraping, eluted from the silica gel by ether and derivatized to methyl ester by treatment with diazomethane. Tests with standard saturated and monounsaturated aldehydes confirmed the validity of the oxidation, and FAME profiles from oxidation of the aldehydes released from the bovine heart PC closely resembled the profiles of the underivatized aldehydes. Finally, the phosphorus-containing species–still at the origin–could be separated from one another by developing the plate 7 cm with chloroform/methanol/water (65:25:4, v/v/v). The resulting spots could then be visualized by Phospray (Supelco, Bellefonte, PA), scraped and analyzed for phosphorus to determine the plasmalogen content of the original lipid fraction.

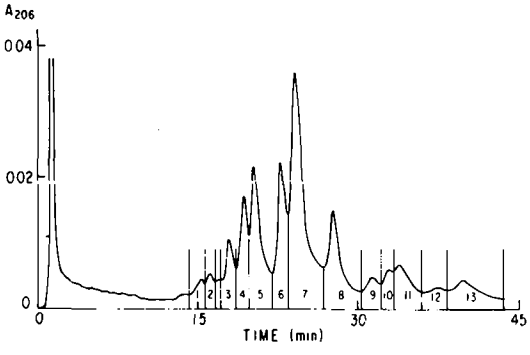

FIG. 1. RP-HPLC of bovine heart PC (0.26 μmol). Column: Beckman Ultrasphere ODS; conditions: cf. Experimental; peak numbers correspond to fraction numbers in Table 1.

Acyl groups were determined by KOH/methanol derivatization to FAME by a microcolumn technique (24), followed by GC analysis (19).

TABLE 1

Lipid Analysis of Bovine Heart PC Fractions Separated by RP-HPLC

Fraction (peak number[a])	Recovered PC[b] (%)	Plasmalogen content[c] (%)	Fraction (peak number)	Recovered PC (%)	Plasmalogen content (%)
1	1.2	5.6	8	18.1	31
2	5.6	33	9	7.3	45
3	4.3	41	10	4.3	18
4	5.2	44	11	6.5	74
5	15.7	15	12	2.5	54
6	7.2	76	13	3.6	52
7	18.4	85	Total	100	47

[a]Cf. Figure 1.

[b]Mol percentage of total PC, based on phosphorus analysis.

[c]Mol percentage of fraction, based on phosphorus analysis (cf. Experimental).

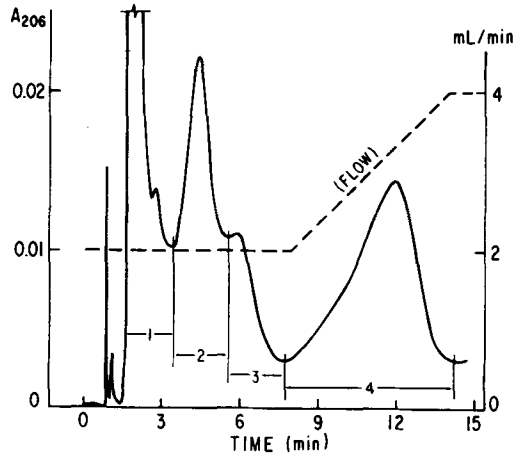

FIG. 2. Argentation HPLC of bovine heart PC (0.26 μmol). Column: ES Chromegabond P-SCX; Ag/PC = 86:1; other conditions–cf. Experimental; peak numbers correspond to fraction numbers in Tables 2 and 3.

RESULTS AND DISCUSSION

Figure 1 shows the trace from a reversed-phase separation of bovine heart PC. Table 1 provides the information on lipid analysis of the components labeled in this figure.

Although the pattern of plasmalogen elution from this column was unpredictable, the fraction giving the largest detector response (peak 7) was highly enriched in plasmalogen content. Together with the preceding fraction (peak 6), RP-HPLC allowed 26% of the total PC to be isolated with an 82% plasmalogen content.

TABLE 2

Lipid Analysis of Bovine Heart PC Fractions from Argentation HPLC[a]

Fraction[b]	Recovered PC[c] (%)	Plasmalogen content[d] (%)
1	10	19
2	41	19
3	8	78
4	30	75
5 (postwash)	11	58
Total	100	47

[a]Run on ES column.

[b]Cf. Figure 2.

[c]Mol percentage of total PC, based on phosphorus analysis.

[d]Mol percentage of fraction, based on phosphorus analysis (cf. Experimental).

Because the present research sought a chromatographic procedure that allowed isolation of plasmalogen by virtue of the unique alk-1-enyl ether functionality of that class of lipids, greater attention was given to charge-transfer HPLC than to RP-HPLC. The ideal electrophile for HPLC would have to interact specifically with the alk-1-enyl double bond, but only in a reversible way, and without catalyzing any reaction of the double bond with the mobile phase. The electrophile would best be immobilized on a strong cation exchange column. Initial experiments with such argentation-HPLC showed that bovine heart PC could be fractionated on a 5-micron column (Macherey-Nagel) into 4 major fractions. This separation

TABLE 3

Moles of Acyl and Alk-1-enyl Chain per 50 Mol of Bovine Heart PC[a]

C=C per chain: Type of chain:	0 acyl	1 acyl	1 alkenyl	2 acyl	3 acyl	4 acyl	5 acyl	Total chain	Average C=C per chain
Fraction									
Total PC	28.9	16.8	23.5	21.6	3.3	4.9	1.1	100	1.2
Plasmalogen	1.7	6.1	23.5	11.4	1.7	2.3	0.5	47	1.5
Other PC	26.3	13.6	0.0	10.3	1.3	1.3	0.2	53	0.8
Sum	28.0	19.7	23.5	21.6	2.9	3.6	0.7	100	1.1
1	5.1	3.9	1.0	0.1	0	0	0	10	0.5
2	15.0	8.6	3.9	13.4	0	0	0	41	1.0
3	1.3	2.5	3.1	0.7	0.1	0.2	0	8	1.1
4	2.0	1.1	11.3	12.6	2.1	0.9	0	30	1.6
5	1.3	0.2	3.2	0.5	1.0	4.2	0.7	11	2.5
Sum	24.7	16.4	22.4	27.3	3.2	5.3	0.7	100	1.3

[a]On ES column.

resulted from adjusting the silver ion load to a silver/lipid ratio (Ag/PC) of 86:1. (PC did not elute from a column that had been saturated with silver ion [Ag/PC = 4000:1].) TLC analysis showed that each fraction was progressively more enriched in plasmalogen content. Nevertheless, very high column pressures (ca. 4,000 lb/sq in.) were necessary. Therefore, this column, with particle size of 5 microns, was replaced with another one (ES) of 10-micron particle size. The optimized separation on this ES column still was achieved with Ag/PC = 86:1, though the elution pattern shown in Figure 2 differed from the pattern seen from the earlier column. Required adjustments of solvent composition and flow rate, and the differences in elution patterns from column to column no doubt reflected nonuniform distribution of silver ion during partial argentation of an existing column. Better consistency may be possible by packing a column with support that already has been partially argentated. However, this would require testing several columns that differ in their silver load. Table 2 gives the lipid analysis for the separation shown in Figure 2. The initially eluting fractions (1 and 2) were low in plasmalogen content, whereas the later ones (3-5) were rich in it. Fraction 5 was the postwash, the lipid that was released by treatment with 1-hexene.

To understand the processes that led to plasmalogen enrichment of the later fractions, the recovered lipids were analyzed for their acyl and alk-1-enyl content. Direct GC analysis of aldehydes liberated from the fractions showed essentially no difference in aldehyde composition from fraction to fraction. Oxidation and derivatization to FAME showed that the aldehydes were mainly palmitaldehyde with some stearaldehyde and olealdehyde, in agreement with published analyses of other bovine heart plasmalogen (25). Unsaturation content was 1.06 double bond/alk-1-enyl chain, including the enol ether functionality.

Acyl analysis, on the other hand, showed great differences from fraction to fraction. Detailed acyl profiles were recalculated from a weight basis (the flame ionization response of the GC) to a molar basis. The molar amounts then were grouped according to double-bond content to show the distribution of unsaturation fraction by fraction. Table 3 shows this distribution, in units of mol of acyl and alk-1-enyl chain per 50 mol of bovine heart PC (50 mol of PC gives rise to 100 mol of chain). The distribution of the total PC may be compared with the summations from the PC fractions. Concordance is adequate, given the experimental error of the procedures used to generate the data.

The last column of Table 3, average C=C per chain, was calculated as shown in the following example: the first HPLC fraction was 10% of the total lipid, or 5.0 mol out of 50 mol of total lipid. These 5 mol gave rise to 10 mol of chain, of which 5.1 mol were saturated acyl groups (no C=C), 3.9 mol were monoenoic acyl groups (1 C=C), 0.9 mol were alk-1-enyl groups (1.06 C=C) and 0.1 mol were dienoic acyl groups (1 C=C), 0.9 mol was alk-1-enyl groups (1.06 C=C) and 0.1 mol was dienoic acyl groups (2 C=C). These 10 mol of chain therefore averaged 0.5 mol of C=C per mol of chain [(5.1 × 0) + (3.9 × 1) + (0.9 × 1.06) + (0.1 × 2) mol of C=C/10 mol of chain], as indicated in the last column of Table 3. The table also shows that the saturated acyl chains originated mainly in the nonplasmalogen PC, and that the plasmalogen had about double the unsaturation of the nonplasmalogen (1.5 vs 0.8 C=C per chain). Most importantly, the table also shows that the retention times of the fractions increased with the degree of overall unsaturation (0.5-2.5 C=C per chain). The last fraction was so unsaturated, in fact, that 1-hexene was necessary to displace it from the column.

Thus, argentation-HPLC succeeded in fractionating bovine heart PC into several fractions based on overall unsaturation, and not on plasmalogen content alone. Nevertheless, because the plasmalogen was also highly unsaturated in acyl content, it tended to be retained on the column better than the nonplasmalogen. In fact, in the experiment illustrated by Figure 2 and Tables 2 and 3, starting PC, 47% of which was plasmalogen, gave a 49% yield of PC enriched to 72% plasmalogen (combined fractions 3-5).

The influence of the overall unsaturation on the fractionation of bovine heart PC by argentation-HPLC thus confirms the earlier findings by argentation TLC (14,15). Furthermore, the findings concur with a report on the equilibrium of model olefins with silver nitrate surfaces, which found that "despite a possible influence of a large polarization of the carbon-carbon double bond in [alk-1-enyl] ethers, the equilibrium data obtained for these compounds have manifested general features similar to those already recognized for olefins" (26).

Variations on argentation HPLC perhaps could lead to a system more specific for the alk-1-enyl functionality. Mercuric ion, for example, is well known as a coordinator with this functionality, but in aqueous systems leads to hydrolysis and aldehyde formation. Because

water is a mandatory component of mobile phases for the elution of phosphatides from silica-based HPLC columns, mercuration HPLC by the systems used for argentation HPLC would seem to be precluded. A recent report, however, showed that mercuric ion-induced hydrolysis of plasmalogens is inhibited at lower temperatures. Thus, a chloroform solution of plasmalogen-rich lipid in contact with an aqueous solution of mercuric acetate at 0 C transferred Hg II ion into the organic phase; titration of the transferred Hg II ion correlated well with the plasmalogen content (3). This encouraged us to test mercuration HPLC of bovine heart PC at reduced temperatures. The ion exchange column, pretreated with mercuric acetate, was routed through an ice-water bath. Elution of injected bovine heart PC gave an initial fraction composed almost entirely of nonplasmalogen PC. This fraction was followed by 2-acylglycerophosphocholine, the residue of plasmalogen PC. Similar results occurred when mercuric ion was replaced by phenylmercuric ion. Thus, on-column hydrolysis of the plasmalogen by mercuric species could not be suppressed by operating at low temperature. The absence of a purple color when treating eluate from the mercuric columns of the present work with s-diphenylcarbazone indicated that these columns did not suffer leaching of the ions during treatment with the bovine heart PC. Such was not the case, however, with columns treated with Pd II ion; s-diphenylcarbazone detected leaching on introducing the PC to the column.

CONCLUSIONS

Two methods have been demonstrated for isolating PC fractions enriched in plasmalogen. From bovine heart PC containing 47% plasmalogen, RP-HPLC gave a 26% yield of PC enriched to 82% plasmalogen, though the separation of PC fractions did not use the unique functionality of the plasmalogen—the alk-1-enyl group. Argentation HPLC of the same PC gave a 49% yield of PC enriched to 72% plasmalogen. Although the electron-rich alk-1-enyl group offered the potential for selective complexation with silver ion, the total unsaturation content of the PC contributed to the actual separation. Enrichment of the late-eluting fractions in plasmalogen occurred because the plasmalogens were more highly unsaturated than their nonplasmalogen counterparts.

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REFERENCES

1. Huisgen, R. (1977) Acc. Chem. Res. 10, 117-124.
2. Smirnov, A.I., Deryabina, G.I., Kalabin, G.A., Istomin, B.I., Chipanina, N.N., Petrova, T.L., and Bairov, V.V. (1975) Bull. Acad. Sci. USSR, Div. Chem. Sci. 161-163; Izv. Akad. Nauk SSSR, Ser. Khim. 172-174.
3. Carey, E.M. (1982) Lipids 17, 656-661.
4. Renkonen, O. (1968) Biochim. Biophys. Acta 152, 114-135.
5. Totani, N., and Mangold, H.K. (1981) Microchim. Acta 1, 73-78.
6. Nakagawa, Y., and Horrocks, L.A. (1983) J. Lipid Res. 24, 1268-1275.
7. Blank, M.L., Cress, E.A., Lee, T., Stephens, N., Piantidosi, C., and Snyder, F. (1983) Anal. Biochem. 133, 430-436.
8. Viswanathan, C.V. (1974) J. Chromatogr., Chromatogr. Rev. 98, 129-155.
9. Arvidson, G.A.E. (1965) J. Lipid Res. 6, 574-577.
10. Arvidson, G.A.E. (1967) J. Lipid Res. 8, 155-158.
11. Arvidson, G.A.E. (1968) Eur. J. Biochem. 4, 478-486.
12. Nakayama, F., and Kawamura, S. (1967) Clin. Chim. Acta 17, 53-58.
13. Hopkins, S.M., Sheehan, G., and Lyman, R.L. (1968) Biochim. Biophys. Acta 164, 272-277.
14. Hoevet, S.P., Viswanathan, C.V., and Lundberg, W.O. (1968) J. Chromatogr. 34, 195-201.
15. Viswanathan, C.V., Hoevet, S.P., and Lundberg, W.O. (1968) J. Chromatogr. 35, 113-116.
16. Smith, M., Monchamp, P., and Jungalwala, F.B. (1981) J. Lipid Res. 22, 714-719.
17. Smith, M., and Jungalwala, F.B. (1981) J. Lipid Res. 22, 697-704.
18. Patton, G.M., Fasulo, J.M., and Robins, S.J. (1982) J. Lipid Res. 23, 190-196.
19. Maxwell, R.J., and Marmer, W.N. (1983) Lipids 18, 453-459.
20. Adlof, R.O., Rakoff, H., and Emken, E.A. (1980) J. Am. Oil Chem. Soc. 57, 273-275.
21. Adlof, R.O., and Emken, E.A. (1980) J. Am. Oil Chem. Soc. 276-278.
22. Vaskovsky, V.E., Kostetsky, E.Y., and Vasendin, I.M. (1975) J. Chromatogr. 114, 129-141.
23. Horrocks, L.A. (1968) J. Lipid Res. 9, 469-472.
24. Marmer, W.N. (1981) Lipids 13, 835-839.
25. Curstedt, T. (1977) Biochim. Biophys. Acta 489, 79-88.
26. Fueno, T., Kajimoto, O., Okuyama, T., and Furukawa, J. (1968) Bull. Chem. Soc. Jpn. 41, 785-790.

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COMMUNICATIONS

Acyl-Acyl Carrier Protein as Substrate of the Acyltransferase of Rat Liver Microsomes

E.L. PUGH and M. KATES, *Department of Biochemistry, University of Ottawa, Ottawa, Ontario, Canada K1N 9B4*

ABSTRACT

Acyl-acyl carrier protein (acyl-ACP) can serve as well as acyl-CoA as substrate of the 1-acyl-*sn*-glycero-3-phosphocholine (1-acyl-GPC) acyltransferase of rat-liver microsomes. The product of the acylation with either thioester substrate is predominantly phosphatidylcholine (PC) (92-95%). The acyl-group transferred from either myristoyl-CoA or myristoyl-ACP is located at the C-2 position of the phospholipid (PL). The apparent Km values for the myristoyl-CoA and myristoyl-ACP were 46 μM and 63 μM, and the corresponding apparent Vmax values were 1.0 and 1.6 nmol/min/mg. The rate of acylation with the acyl-ACP was unaffected by the addition of free CoA-SH. These data suggest that acyl-CoA and acyl-ACP are transferred to 1-acyl-GPC by the same or similar enzyme systems.

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INTRODUCTION

Naturally occurring phospholipids (PL) usually have a saturated fatty acid at position 1 and an unsaturated fatty acid at position 2 of the *sn*-glycerol-3-phosphate backbone (1). In animal systems, the incorporation of unsaturated fatty acids into the 2 position of PL is catalyzed by acyl-CoA:1-acyl-*sn*-glycero-3-phosphate (1-acyl-GP) and acyl-CoA:1-acyl-*sn*-glycero-3-phosphocholine (1-acyl-GPC) acyl transferase systems. In liver, monoenoic and dienoic fatty acids may be incorporated into PL mainly by the 1-acyl-GP acyltransferase system whereas arachidonate and other long-chain polyunsaturated acids may be esterified mainly by the 1-acyl-GPC acyltransferase system (2).

The acylation specificity of the 1-acyl-GPC acyltransferase has also been studied in the presence of mixtures of unsaturated acyl-CoAs (3-6), and under these conditions the positional distribution of fatty acids in PL observed in vitro is similar to the distribution in vivo. In a recent study (7), no effect on the activity of liver microsomal 1-acyl-GPC transacylase was observed in rats fed a corn-oil or coconut-oil diet compared with those fed a control, fat-free diet.

A major complication in evaluating these data with acyl-CoA substrates is that they are potent inhibitors, above their critical micelle concentration, of the acyltransferase because of the formation of detergent micelles (8). This property makes including serum albumin in the assay necessary to overcome the detergent effect of the substrate. This difficulty has led us to evaluate an acyl-acyl carrier protein (acyl-ACP) that does not exhibit detergent properties, as substrate for the acyltransferase, in comparison with the corresponding acyl-CoA.

Furthermore, determining whether the 1-acyl-GPC acyltransferase could use acyl-ACP as acyl donor in animal systems, as has been demonstrated in bacterial (9) and plant (10) systems, for the acylation of glycerophosphate is of interest.

This communication reports that an acyl-ACP (myristoyl-ACP) can serve as well as the corresponding acyl-CoA (myristoyl-CoA) as substrate for the 1-acyl-GPC acyltransferase of rat-liver microsomes.

MATERIALS AND METHODS

[9,10-^{3}H]Myristoyl-CoA (0.052 Ci/mmol) and [9,10-^{3}H]myristoyl-acyl carrier protein (0.85 Ci/mmol) were the generous gift of Dr. E. Do, Lipid Group, New England Nuclear (Boston, MA). The myristoyl-ACP is a synthetic product derived from ACP of *Escherichia coli*. Concentrations of acyl-CoA and acyl-ACP were determined by measuring the release of free CoA-SH or ACP-SH after alkaline hydrolysis according to the method of Ray and Cronan (11). These values for acyl-CoA and acyl-ACP agreed well with values obtained by calculation from the specific activity of the radioactive myristate used in the preparations. The authenticity of the acyl-CoA and acyl-ACP was determined from the ultraviolet (UV) absorption spectra. The acyl-CoA had an absorbance ratio (A_{232}/A_{260}, thiol absorption/adenine absorption) of 0.61 (theoretical, 0.573) (12). The acyl-ACP and the ACP-SH released after alkaline hydrolysis had an absorption peak at

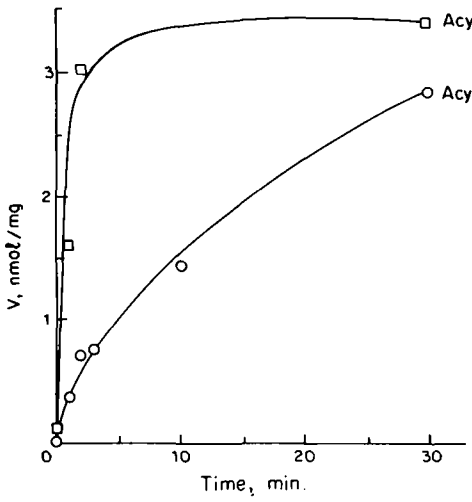

FIG. 1. Time course of 1-acyl-*sn*-glycero-3-phosphocholine acyltransferase with either myristoyl-CoA (□) or myristoyl-acyl carrier protein (○) as acyl donor. Assay conditions are described in the text.

275 nm, which has been shown to be characteristic of acyl carrier protein (13).

Rats were starved and refed a fat-free diet as described by Strittmatter et al. (14) and liver microsomes were prepared as described previously (15). 1-Acyl-GPC acyltransferase activity was measured by incorporating either [9,10-^{3}H]myristoyl-CoA or [9,10-^{3}H]myristoyl-ACP into phosphatidylcholine (PC) as described previously (7). The complete reaction mixture (1.0 ml) contained 0.1 M Tris-HCl (pH 7.2), 50 μM 1-palmitoyl-*sn*-glycero-3-phosphocholine, 400 μg microsomal protein, 1 mg bovine serum albumin and either 1-3 μM [9,10-^{3}H]14:0-CoA (0.052 Ci/mmol) or 1-3 μM [9,10-^{3}H]14:0-ACP (0.85 Ci/mmol). Incubations were carried out at room temperature for the time periods indicated in the table and figure legends. The reaction was terminated by extracting the lipids by the Bligh and Dyer procedure (16). PL were isolated by silicic acid column chromatography and individual species examined by thin layer chromatography (TLC), as described previously (7).

The positional distribution of [^{3}H]myristate incorporated into PC was determined by treatment with phospholipase A_2 from *Naja naja* venom (17) and protein was determined by the method of Lowry et al. (18).

RESULTS AND DISCUSSION

1-Acyl-*sn*-glycero-3-phosphocholine acyltransferase activity was demonstrated in rat-liver microsomes with either myristoyl-CoA or myristoyl-ACP serving as acyl donor. The species of acyl donor used had an influence on the time course of acyltransferase activity (Fig. 1). Under conditions known to allow maximum enzyme activity (7), but in the presence of serum albumin, incorporation of [^{3}H]myristoyl-CoA into PC proceeded linearly for ca. 2-3 min. When [^{3}H]myristoyl-ACP was substituted for myristoyl-CoA in the reaction mixture, the reaction also proceeded linearly for ca. 2-3 min but a somewhat slower reaction was observed over a 30 min period. Under the same conditions, but in the absence of serum albumin, no change was observed in the reaction rates (data not shown). Furthermore, the addition of CoA-SH to the reaction mixture had no effect on the rate of acylation with myristoyl-ACP as substrate (data not shown).

The major lipid formed with both myristoyl-CoA and myristoyl-ACP as substrate was PC

TABLE 1

Products of the Acylation of 1-Acyl-*sn*-glycero-3-phosphocholine with Myristoyl-CoA and Myristoyl-ACP by Acyltransferase of Rat-Liver Microsomes[a]

Acyl donor	Time of incubation	Phosphatidylcholine	Free fatty acid
	Min	nmol of Lipids/mg protein	
Myristoyl-CoA	1	1.60 (93.8%)[b]	0.10 (5.9%)
Myristoyl-CoA	3	3.01 (95.1%)	0.15 (4.7%)
Myristoyl-ACP	1	0.35 (92.0%)	0.03 (7.9%)
Myristoyl-ACP	3	0.76 (93.8%)	0.05 (6.0%)

[a]The reaction mixture was as described in the text. Results are the means of duplicate determinations with deviations of ±2% and have been corrected for background.

[b]The number in parenthesis represents the percentage of total [^{3}H]lipid product.

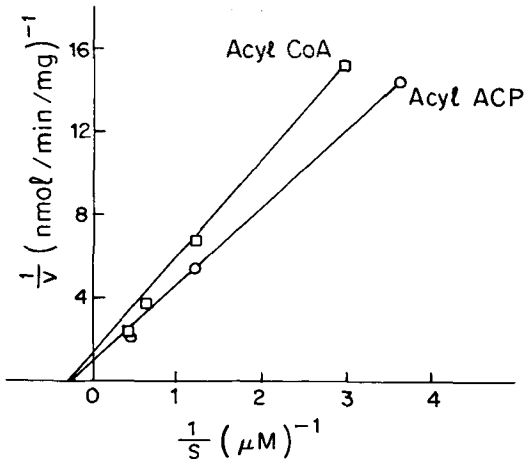

FIG. 2. Myristoyl-CoA and myristoyl-acyl carrier protein dependence of 1-acyl-*sn*-glycero-3-phosphocholine acyltransferase activity. The assay conditions were as described in the text except that various concentrations of myristoyl-CoA (□) and myristoyl-ACP (○) were used. Incubations were at room temperature for 3 min.

(Table 1). With both the CoA and ACP substrates, some free fatty acid (FFA) (5-8% of the total lipids) was formed, presumably by the action of the fatty acyl-CoA hydrolase, which is known to be active in these microsomes (7). The relative distribution of these 2 lipids was about the same with both substrates and constant throughout the linear portion of the time curve. Only trace amounts of other lipids including lysophosphatidylcholine, phosphatidylethanolamine (PE) and phosphatidylserine-phosphatidylinositol (PS-PI) were found (<0.2%).

The PC product recovered from experiments using [^{3}H]myristoyl-CoA or [^{3}H]myristoyl-ACP as substrate was treated with snake venom phospholipase A_2 to determine the position of incorporation of radioactivity into PL. With the acyl-CoA substrate, the radioactivity was found to be incorporated predominantly at the C-2 position (97%) with the remainder being incorporated at the C-1 position (3%) of the PL. Note that in animal systems, long-chain saturated (16:0, 18:0) acyl-CoA are incorporated specifically into the C-1 position (1). With the acyl-ACP substrate, the radioactivity was incorporated almost exclusively at the C-2 position (>99%).

Myristoyl-CoA and myristoyl-ACP have similar activities as substrates for 1-acyl-GPC acylation (Fig. 2). The apparent Km for myristoyl-CoA was ca. 46 μM whereas the apparent Km of myristoyl-ACP was ca. 63 μM. Apparent Vmax values for myristoyl-CoA and myristoyl-ACP were 1.0 and 1.6 nmol/min/mg.

The above data thus show that an acyl-ACP –myristoyl-ACP, as well as the analogous acyl-CoA–myristoyl-CoA, can serve as substrate for the 1-acyl-GPC acyltransferase of rat-liver microsomes. These results suggest that the 2 thioesters may be transferred to the lipid acceptor by the same or similar enzyme system. This conclusion is supported by the very similar Km values of the acyl-ACP and acyl-CoA substrates. The alternate route in which the acyl group is transferred from acyl-ACP to endogenous CoA and then to the lipid acceptor is unlikely because the addition of CoA does not affect the rate of acylation. The finding that with both myristoyl-CoA and myristoyl-ACP, the myristoyl group is specifically incorporated into the 2-position usually reserved for unsaturated acyl chains is noteworthy. Further investigation of this observation would be worthwhile.

The present results also show that at the low substrate concentrations used (1-3 μM), no detergent effect of the myristoyl-CoA was observed. However, at high substrate concentrations, where long-chain acyl-CoA have inhibitory detergent properties but acyl-ACP do not (8), the acyl-ACP could be used as a model substrate for the 1-acyl-GPC acyltransferase.

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REFERENCES

1. van Deenen, L.L.M. (1965) in Progress in the Chemistry of Fats and Other Lipids (Holman, R.T., ed.) Vol. 8, Part 1, pp. 1-115, Pergamon Press, Oxford and New York.
2. Hill, E.E., and Lands, W.E.M. (1968) Biochim. Biophys. Acta 152, 645-648.
3. Okuyama, H., and Lands, W.E.M. (1972) J. Biol. Chem. 247, 1414-1423.
4. Okuyama, H., Yamada, K., and Ikezawa, H. (1975) J. Biol. Chem. 250, 1710-1713.
5. Possmayer, F., Scherphof, G.L., Dubbelman, T.M.A.R., van Golde, L.M.G., and van Deenen, L.L.M. (1969) Biochim. Biophys. Acta 176, 95-110.
6. Lands, W.E.M., Inoue, M., Sugiura, Y., and Okuyama, H. (1982) J. Biol. Chem. 257, 14968-14972.
7. Pugh, E.L. and Kates, M. (1984) Lipids 19, 48-55.
8. Kito, M. and Pizer, L.I., J. Bacteriol. 97:1321-1327 (1969).
9. Green, P.R., Merrill, A.H., Jr., and Bell, R.M. (1981) J. Biol. Chem. 256, 11151-11159.

10. Frentzen, M., Heinz, E., McKeon, T.A., and Stumpf, P.K. (1983) Eur. J. Biochem. 129, 629-636.
11. Ray, T.K., and Cronan, J.E., Jr. (1975) J. Biol. Chem. 250, 8422-8427.
12. McKean, M.L., Smith, J.B., and Silver, M.J. (1982) J. Biol. Chem. 257, 11278-11283.
13. Pugh, E.L., and Wakil, S.J. (1965) J. Biol. Chem. 240, 4727-4733.
14. Strittmatter, P., Spatz, L., Corcoran, D., Rogers, M.J., Setlow, B., and Redline, R. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 4565-4569.
15. Pugh, E.L., and Kates, M. (1977) J. Biol. Chem. 252, 68-73.
16. Bligh, E.C., and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911-919.
17. Pugh, E.L., Kates, M., and Szabo, A.G. (1982) Chem. Phys. Lipids 30, 55-69.
18. Lowry, O.H., Rosebrough, M.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.

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Distribution of Antithrombin III and Glucosylceramide in Human Plasma Lipoproteins and Lipoprotein Deficient Plasma

SUBROTO CHATTERJEE[a]*, WILLIAM R. BELL[b] and PETER O. KWITEROVICH, JR.[a,b],
Lipid Research-Atherosclerosis Unit, Departments of [a]Pediatrics and [b]Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

ABSTRACT

We have investigated the distribution of antithrombin-III and glucosylceramide (Glc-Cer) in human plasma, plasma lipoproteins and lipoprotein-deficient plasma. Antithrombin III activity was measured employing immunochemical and biological assays. Glc-Cer was quantified by gas liquid chromatography (GLC). Whole plasma contained 145 μg antithrombin III/ml plasma, all of which was associated with the lipoprotein-deficient plasma (d > 1.25 g/ml). Whereas, most if not all the plasma GlcCer was associated with plasma low density lipoproteins (LDL) (d-1.022-1.055 g/ml) and high density lipoproteins (HDL) (d-1.063-1.25). GlcCer was not found in the lipoprotein-deficient plasma. We conclude that GlcCer on lipoproteins does not contribute to antithrombin III activity. Moreover, the absence of GlcCer in lipoprotein-deficient plasma does not impair antithrombin-III activity.
Lipids 19: 363-366, 1984.

INTRODUCTION

Antithrombin III is a α2-plasma protein which progressively inactivates thrombin (1) and several serine proteases that participate in the coagulation process (2). Deficiency of antithrombin III may be responsible for recurrent thrombosis (3). In contrast, excessive bleeding occurs when the levels of antithrombin III are increased (4). Antithrombin III is a glycoprotein (Mr≈60,000) with 4 identical N-glycosidically linked carbohydrate chains per molecule (5). Recently, glucosylceramide was found to complex tightly with antithrombin III (6). The latter finding is of considerable interest as most if not all the glycosphingolipid, including glucosylceramide, is associated with lipoproteins (7), and antithrombin III complexed with GlcCer may produce anticoagulative properties. In addition, these studies implied glycosphingolipid function in the blood coagulation process. We therefore investigated the distribution of antithrombin III and GlcCer in normal human plasma, various plasma lipoproteins and lipoprotein-deficient plasma. Our observations indicate that antithrombin III and GlcCer are not tightly complexed and that GlcCer is not required for antithrombin III activity.

MATERIALS AND METHODS

Antithrombin III

Human antithrombin III and goat anti-human III antibody were obtained from Alpha Therapeutic Corporation (Los Angeles, CA) and Cutter Laboratories, Inc., Berkeley, CA. Normal human plasma, plasma lipoproteins and lipoprotein-deficient plasma were prepared exactly as described previously (7).

Measurement of Antithrombin III Activity

Antithrombin III activity was measured by immunochemical and biological assays described previously (8,9). These studies were performed on fresh plasma or newly reconstituted lyophilized antithrombin III (human). Trisodium citrate dehydrate, 3.8%, 1 vol of which was mixed with 9 vol of whole blood, was the anticoagulant employed. Heparin was not in any of the specimens assayed. All specimens were assayed in triplicate.

Measurement of Glucosylceramide

Total lipids in antithrombin III, whole plasma, plasma lipoproteins and lipoprotein-deficient plasma were isolated by extraction with organic solvents (7). GSL were isolated from the total lipid extracts by silicic acid column chromatography, purified by thin layer chromatography (TLC) and quantified by gas liquid chromatography (GLC), exactly as described previously (7). Mannitol was used as an internal standard.

RESULTS

Antithrombin III levels in Human Plasma, Plasma Lipoproteins and Lipoprotein-Deficient Plasma

The mean level of human plasma antithrom-

*To whom all correspondence should be addressed.

TABLE 1

Distribution of Antithrombin III and Glucosylceramide in Human Plasma, Plasma Lipoproteins and Lipoprotein-Deficient Plasma

	Plasma	Plasma Lipoproteins				Lipoprotein-deficient plasma
		VLDL	IDL	LDL	HDL	
Antithrombin III (μg/ml)	145 (139-152)	0	0	0	0	162.5 (145-180)
Glucosylceramide (nmol/ml)	8 (6-10)	1 (0.7-1.3)	0	4.5 (3.5-5.5)	2.5 (2-3)	0 0

Fasting blood was collected from 2 males, S.C. and R.W. Total plasma, plasma lipoproteins and lipoprotein-deficient plasma d > 1.25 g/ml was prepared as described previously (7). Plasma lipoproteins and lipoprotein-deficient plasma samples were analyzed to determine antithrombin III (8,9) and glucosylceramide levels. The results are presented as mean values and the individual values are presented within parentheses. VLDL, very low density (prebeta) lipoproteins (d < 1.006 g/ml); IDL, intermediate density lipoproteins (d 1.006-1.019 g/ml); LDL, low density (beta) lipoproteins (d 1.022-1.055 g/ml); HDL, high density (alpha) lipoproteins (d 1.063-1.25 g/ml).

bin III was 145 μg/ml (Table 1). No antithrombin III was found in the plasma lipoproteins. In sharp contrast, the mean level of antithrombin III in lipoprotein-deficient plasma was 162 μg/ml. Thus, all the antithrombin III present in whole plasma was recovered in the lipoprotein-deficient plasma. The values obtained with the biologic functional assay were identical to those obtained with the immunologic technique.

Glucosylceramide Levels in Human Plasma, Plasma Lipoproteins and Lipoprotein-Deficient Plasma

The mean level of GlcCer in human plasma was 8 nmol/ml. The mean levels (nmol/ml) of GlcCer in very low density lipoprotein (VLDL), low density lipoprotein (LDL) and high denisty lipoprotein (HDL) were 1, 4.5 and 2.5. GlcCer was not found in plasma intermediate density lipoproteins (IDL) and in lipoprotein-deficient plasma. Antithrombin III obtained commercially contained 1.4 nmol GlcCer/20 mg protein. Thus, most if not all the GlcCer present in whole plasma is associated with the human plasma lipoproteins. Antithrombin III contained very small amounts of the GlcCer in relation to whole plasma.

DISCUSSION

The major findings of these studies are: first, antithrombin III is associated with the lipoprotein-deficient plasma; second, little if any glucosylceramide is tightly associated with antithrombin III; and third, antithrombin III activity does not require glucosylceramide.

We have shown previously that most if not all the normal human plasma glycosphingolipids (GSL) are associated with the human plasma lipoproteins (7). In particular, plasma LDL, which carry most of the plasma cholesterol, are enriched in various plasma GSL including GlcCer (10-12). Moreover, GSL distribution studies in lipoproteins obtained from patients with Fabry's disease (α-galactosidase-A-deficiency) and Gaucher's disease (β-glucosidase deficiency) have revealed enrichment of trihexosylceramide (Gl_{3a}) and glucosylceramide, respectively, in LDL (11,13). No GSL were found in lipoprotein-deficient plasma.

Recently, glucosylceramide was found to be tightly associated with antithrombin III, and glucosylceramide was implicated in the anticoagulative action of antithrombin III (6). Based on these findings, we rationalized that human plasma lipoproteins, which contain most if not all the GlcCer, should also be enriched with antithrombin III. Thus, exploring the role of lipoproteins in the pathogenesis of atherosclerosis may be possible.

We therefore fractionated normal human plasma by KBr density gradient centrifugation. The resultant plasma lipoproteins and lipoprotein-deficient plasma were dialyzed and the levels of antithrombin III and glucosylceramide were measured. We found a clear dichotomy of distribution of antithrombin III and GlcCer among plasma lipoproteins and lipoprotein-deficient plasma. Antithrombin III was completely recovered in the lipoprotein-deficient plasma and none was detectable in the plasma lipoprotein fractions. In contrast, as expected from our previous work and that of other investigators (7,10-12), GlcCer levels in the various plasma lipoproteins accounted for all the GlcCer present in human plasma. Moreover, GlcCer was not found to associate with the

lipoprotein-deficient plasma. A similar observation was found by Dawson and Oh (13) in patients with Gaucher's disease who have elevated GlcCer levels in plasma and LDL. Commercially available antithrombin III contained 1.4 nmol GlcCer/20 mg protein. Considering that the concentration of antithrombin III in plasma is ca. 14 mg/100 ml and the levels of glucosylceramide is 800 nmol/100 ml, and as only 1.4 nmol GlcCer is associated with 20 mg antithrombin III, very little, if any, of the plasma GlcCer is trightly associated with antithrombin III.

Our findings are in contrast to a previous report (6) in which practically all of the plasma GlcCer was considered to associate tightly with antithrombin III and that antithrombin III serves as a carrier for GlcCer in plasma. Our data clearly suggest that plasma lipoproteins, particularly LDL, are the predominant carriers of plasma GlcCer, not antithrombin III. On the other hand, antithrombin III is not strongly associated with any plasma lipoprotein and hence is found in the lipoprotein-deficient plasma.

Several reasons may be advanced for the discrepancy between the observations of Danishefsky and coworkers (6) and the data presented here. Lipoproteins are known to be a possible source of contamination in the isolation of antithrombin III (14). Danishefsky et al. (6) used Method B of Thaler and Schmer (14) to isolate human antithrombin III (except that the affinity chromatography step was performed with heparin/aminohexyl Sepharose). This method employs precipitation of proteins from plasma with polyethylene glycol, followed by heparin-agarose chromatography (14). Two sources of contamination are possible with plasma lipoproteins. When the precipitate from polyethylene glycol is resolubilized and the protein reprecipitated with ammonium sulfate, a small floating lipoprotein layer is present in the supernatant after centrifugation. Although the ammonium sulfate step is reported to remove contamination with plasma lipoproteins, some material (that may be LDL), can still be seen at the origin of sodium dodecyl sulfate polyacrylamide gels (14). Second, after only 3 preparations of antithrombin III have been applied to the heparin affinity columns, the columns need to be washed carefully to remove all components nonspecifically bound, especially plasma lipoproteins, which stick tightly to such columns and are not removed by the usual wash procedures (14). In addition to the above approach, Danishefsky et al. (6) also used alternate methods to remove the glucose derivative from antithrombin III but were unable to reduce the proportion of glucose present. We used commercially obtained antithrombin III (see Methods). Following "Cohn fractionation," antithrombin III is isolated from the lipoprotein-rich fraction by prolonged heating (10 hr at 60 C) in the presence of high salt concentrations, followed by heparin-Sepharose column chromatography. Since we found little if any glucosylceramide on antithrombin III prepared in this way, the method probably dissociated any lipoprotein-derived glucosylceramide from antithrombin III.

We found, after preparative ultracentrifugation using KBr gradients, that plasma glucosylceramide was associated with the lipoproteins and not with plasma proteins. The plasma protein fraction contained all of the plasma antithrombin III material and also all of the antithrombin III activity. Thus, although ultracentrifugation may have dissociated glucosylceramide and antithrombin III, such a possible effect did not destroy the activity of antithrombin III. In addition, the activity of antithrombin III in unfractionated plasma (which contained lipoproteins) was not greater than that in lipoprotein-deficient plasma, indicating that the presence of lipoproteins did not enhance antithrombin III activity. Danishefsky and coworkers (6) did some exploratory experiments and were unable to find evidence that glucosylceramide was necessary for antithrombin III activity.

Since lipoprotein-deficient plasma did not contain any GlcCer but maintained biological and immunological properties of antithrombin III, we cannot associate any functional role for GlcCer with antithrombin III-related coagulation reactions at our present state of knowledge.

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REFERENCES

1. Marawitz, P. (1905) Die Erg. Physiol. 4, 307-422.
2. Barrowcliffe, T.W., Johnson, E.A., and Thomas, D. (1968) Br. Med. Bull. 34, 143-150.
3. Sas, G., Blasko, G., Banhegyi, D., Jako, J., and Palos, L.A. (1974) Thromb. Diath. Haemorrh 32, 105-155.
4. Robinson, A.J., Aggeler, P.M., McNicol, G.P., and Douglas, A.S. (1967) Br. J. Haematol. 13, 510-527.

5. Franzen, L.E., and Svensson, S. (1980) J. Biol. Chem. 255, 5090-5093.
6. Danishefsky, I., Zweben, A., and Slomiany, B.L. (1980) J. Biol. Chem. 253, 32-37.
7. Chatterjee, S., and Kwiterovich, P.O., Jr. (1976) Lipids 11, 462-466.
8. Mannan, E.F. (1971) in Thrombosis and Bleeding Disorders in Coagulation Disorders (Bond, N.U., Bieller, F.K., Deutsch, E., and Mannan, E.F., eds.), pp. 268-277, Academic Press, Inc., New York.
9. Goitel, S.N., and Wessler, S. (1975) Thrombos. Res. 7, 5.
10. Dawson, G., Kruski, A.W., and Scanu, A.M. (1976) J. Lipid Res. 17, 125-131.
11. Clarke, J.T.R., Stoltz, J.M., and Mulcahey, M.R. (1976) Biochim. Biophys. Acta 431, 317-325.
12. VanDenBergh, F.A.J.T.M., and Tager, J.M. (1976) Biochim. Biophys. Acta 441, 391-395.
13. Dawson, G., and Oh, J.Y. (1977) Clin. Chim. Acta 75, 149-153.
14. Thaler, E., and Schmer, G. (1975) Brit. J. Haematology 31, 233-243.

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Tissue Phospholipid Fatty Acid Composition in the Diabetic Rat

Y.S. HUANG*, D.F. HORROBIN, M.S. MANKU, J. MITCHELL and M.A. RYAN,
Efamol Research Institute, P.O. Box 818, Kentville, Nova Scotia Canada B4N 4H8

ABSTRACT

Tissue phospholipid fatty acid compositions in streptozotocin-induced diabetic rats were studied. The major changes in liver, plasma, erythrocyte and heart were increased proportions of linoleic and dihomo-γ-linolenic acids and a decreased proportion of arachidonic acid. The latter was not significantly changed in phospholipids of kidney, adrenal gland and testis. Skin fatty acids in diabetic rats showed an increase in the proportion of arachidonic acid and a reduction in the proportion of linoleic acid. The fatty acid desaturating activity in diabetes may be regulated differently in different tissues. *Lipids* 19: 367-370, 1984.

INTRODUCTION

Many clinical features in severely diabetic rats, e.g., scaly tails, rough fur and dermal symptoms, are indistinguishable from those in EFA-deficient animals. These similarities are possibly a reflection of an impaired conversion of linoleic acid to arachidonic acid because liver microsomal fatty acid desaturation has been shown to be depressed in experimental diabetic rats (1-6). A decrease in the proportion of arachidonic acid (20:4n-6) and an increase of linoleic acid (18:2n-6) in liver and blood have been frequently reported. Fatty acid compositions of adipose tissues and testes in diabetic rats have also been described (7). Recently, Clark et al. (8) have reported the changes in kidney phospholipid fatty acids and Holman et al. (9) demonstrated the fatty acid compositions of phospholipids of heart, liver, kidney, aorta and serum in diabetic rats. However, detailed data concerning the effect of diabetes on fatty acid profiles and in individual phospholipid components in other tissues are lacking. The purpose of this study was to examine whether previously described changes are also present in other tissues in experimental diabetic rats.

MATERIALS AND METHODS

Male Sprague-Dawley rats weighing 275-325 g were maintained on Purina rat chow and water ad libitum. Rats were made diabetic by a single i.p. injection of 75 mg/kg body weight of streptozotocin; the controls received saline. Two days after injection, blood glucose was estimated using Dextrostix reagent strips (Ames Division, Miles Laboratories Ltd., Rexdale, Ont., Canada). Only rats with blood glucose greater than 250 mg/dl were considered diabetic. Three weeks after diabetic induction, 10 animals from each group were killed under light ether anesthesia by exsanguination. Erythrocytes were separated from plasma and washed with ice-cold saline. Liver, heart, kidney, adrenal gland, testis, skin of the front paw and epididymal fat pad were rapidly excised, washed and frozen. Plasma glucose was measured enzymatically using Cobas Bio centrifugal analyzer (Hoffmann-La Roche Inc., Veudreil, Quebec, Canada).

Plasma and tissue lipids were extracted and total phospholipids (PL) separated from neutral lipids (10). Aliquots of liver and erythrocyte phospholipids were further separated by thin layer chromatography (TLC) (11) to obtain 3 major phospholipid fractions, phosphatidylcholine (PC), phosphatidylinositol and -serine (PI+PS) and phosphatidylethanolamine (PE). Fatty acid methyl esters were prepared with BF-methanol (12) and analyzed by gas liquid chromatography (GLC) (13).

RESULTS

At the end of the experiment, mean (± SEM) plasma glucose concentration was 474.6 (± 36.8) mg/dl in diabetic rats and 135.6 (± 7.2) mg/dl in the controls.

The detailed results showing changes in the fatty acid composition of total phospholipids in liver, plasma, heart, kidney, testis, adipose tissue, skin and adrenal gland are shown in Table 1. Fatty acid levels on PC, PI+PS and PE are shown in Table 2. The changes can be seen in the tables but the following consistent or substantial changes in the diabetic animal are worth emphasizing: (a) changes in saturated fatty acid in PL were relatively small; 16:0 was

*To whom correspondence should be addressed.

TABLE 1

Major Fatty Acid Composition of Total Tissue Phospholipids in Control (C) and Diabetic (D) Rats

	16:0	18:0	18:1	18:2 n6	20:3 n6	20:4 n6	20:5 n3	22:4 n6	22:5 n6	22:5 n3	22:6 n3
Liver											
C	16.5 ± 0.9*	23.2 ± 1.9	6.1 ± 0.7	13.2 ± 1.1	0.9 ± 0.2	28.5 ± 1.6	0.6 ± 0.2	0.3 ± 0.02	0.1 ± 0.02	0.8 ± 0.9	7.7 ± 1.4
D	15.2 ± 1.1[c]	22.9 ± 1.0	7.1 ± 1.8	18.4 ± 1.3[f]	1.8 ± 0.3[f]	21.3 ± 3.3[f]	0.5 ± 0.1	0.2 ± 0.02[f]	0.1 ± 0.02	0.9 ± 0.1	9.7 ± 2.6
Plasma											
C	22.3 ± 1.8	19.9 ± 2.6	5.8 ± 0.7	14.9 ± 1.7	0.7 ± 0.2	28.0 ± 1.8	0.7 ± 0.3	0.8 ± 0.2	-	0.7 ± 0.1	5.0 ± 1.1
D	24.0 ± 1.5	20.5 ± 3.3	5.7 ± 0.6	24.1 ± 3.2[f]	1.5 ± 0.2[f]	16.3 ± 2.3[f]	0.6 ± 0.3	0.6 ± 0.1	-	0.7 ± 0.1	5.4 ± 1.0
Heart											
C	12.2 ± 0.6	21.9 ± 0.3	7.1 ± 0.6	16.9 ± 1.7	0.4 ± 0.05	19.0 ± 1.3	0.3 ± 0.08	0.5 ± 0.07	0.3 ± 0.04	2.1 ± 0.2	16.1 ± 1.1
D	10.4 ± 0.6[f]	22.9 ± 0.5[f]	6.6 ± 0.5	22.2 ± 3.8[e]	0.6 ± 0.09[f]	14.9 ± 1.1[t]	0.2 ± 0.04[f]	0.4 ± 0.03[f]	0.4 ± 0.04	2.4 ± 0.4	16.9 ± 2.0
Kidney											
C	19.1 ± 0.3	18.4 ± 0.4	8.2 ± 0.3	12.5 ± 0.9	1.2 ± 0.1	28.6 ± 1.0	1.0 ± 0.2	1.0 ± 0.2	-	0.6 ± 0.04	3.2 ± 0.2
D	15.8 ± 1.0[f]	20.5 ± 0.7[f]	8.8 ± 0.3	16.6 ± 1.6[f]	1.4 ± 0.17[a]	26.7 ± 1.7	0.7 ± 0.2[f]	0.7 ± 0.2[f]	-	0.5 ± 0.09	3.0 ± 0.3
Testis											
C	33.3 ± 1.0	7.2 ± 0.2	11.1 ± 0.3	4.2 ± 0.4	1.4 ± 0.2	17.1 ± 0.4	0.1 ± 0.02	1.7 ± 0.06	17.9 ± 0.7	1.0 ± 0.1	1.1 ± 0.1
D	33.7 ± 0.8	7.0 ± 0.3	11.1 ± 0.2	4.7 ± 0.3[e]	1.6 ± 0.07	16.1 ± 0.08[e]	0.1 ± 0.02	1.5 ± 0.09[f]	18.5 ± 0.4	0.9 ± 0.06	0.9 ± 0.05[f]
Adipose**											
C	24.7 ± 1.1	3.6 ± 0.3	29.0 ± 1.3	26.8 ± 1.4	-	0.8 ± 0.1	0.4 ± 0.07	-	1.4 ± 0.3	0.5 ± 0.09	1.3 ± 0.2
D	20.6 ± 1.4[f]	5.7 ± 0.6[f]	34.0 ± 1.5[f]	28.9 ± 1.1	0.2 ± 0.03	0.6 ± 0.1[f]	0.2 ± 0.03[f]	-	0.2 ± 0.1[f]	0.4 ± 0.1	1.1 ± 0.2[c]
Skin**											
C	21.2 ± 1.5	6.1 ± 2.3	28.3 ± 1.2	28.0 ± 1.2	0.2 ± 0.01	1.1 ± 0.2	0.2 ± 0.05	-	-	0.1 ± 0.1	0.5 ± 0.2
D	14.3 ± 0.6[f]	12.7 ± 1.5[f]	12.0 ± 5.9[f]	15.6 ± 5.6	0.4 ± 0.07[f]	5.7 ± 1.7[f]	-	-	-	0.2 ± 0.2	1.3 ± 0.3[f]
Adrenal											
C	6.2	34.9	5.8	4.6	0.5	41.8	-	1.3	0.3	0.3	0.6
D	5.0	34.4	4.0	6.3	0.7	46.2	-	1.4	0.1	0.1	0.4

*Percent by weight of total fatty acids. Minor components not shown. Each value represents mean ± SD for 10 rats in each group. Significance of difference between control and diabetic rats were analyzed by Student's *t*-test: [a], $p < 0.05$; [b], $p < 0.025$; [c], $p < 0.02$; [d], $p < 0.01$; [e], $p < 0.005$; [f], $p < 0.001$.

**Total lipids.

TABLE 2

Distribution of Major Fatty Acids in Phospholipid Fractions of Various Tissues in Control (C) and Diabetic (D) Rats. Analysis as in Table 1

	16:0	18:0	18:1	18:2 n6	20:3 n6	20:4 n6	20:5 n3	22:4 n6	22:5 n6	22:5 n3	22:6 n3
						LIVER					
PC-C	16.7 ± 1.5	24.5 ± 1.5	5.1 ± 0.5	10.7 ± 1.4	0.9 ± 0.4	32.1 ± 2.0	0.6 ± 0.2	0.1 ± 0.04	0.1 ± 0.03	0.7 ± 0.1	6.3 ± 1.2
D	17.5 ± 0.7	24.0 ± 1.0	6.2 ± 0.6[f]	18.7 ± 2.0[f]	1.6 ± 0.3[f]	20.9 ± 3.2[f]	0.5 ± 0.1	0.1 ± 0.01	0.1 ± 0.02	0.7 ± 0.2	7.5 ± 2.5
PI-C	2.9 ± 0.4	36.8 ± 0.9	1.5 ± 0.2	2.3 ± 0.4	2.0 ± 0.6	45.4 ± 3.2	0.4 ± 0.1	0.4 ± 0.1	0.2 ± 0.1	1.1 ± 0.3	6.3 ± 1.3
D	2.8 ± 0.5	38.8 ± 1.1[f]	2.2 ± 1.5	4.0 ± 0.9[f]	5.6 ± 0.9[f]	36.3 ± 3.3[f]	0.1 ± 0.03[f]	0.3 ± 0.05	0.2 ± 0.04	1.1 ± 0.2	7.8 ± 1.7[a]
PE-C	14.5 ± 0.7	22.8 ± 1.4	5.9 ± 0.5	11.8 ± 1.4	0.6 ± 0.1	25.8 ± 1.5	0.8 ± 0.3	0.8 ± 0.3	0.1 ± 0.04	1.4 ± 0.2	12.8 ± 2.3
D	13.7 ± 1.4	24.1 ± 0.8[b]	5.5 ± 0.7	13.0 ± 1.5	1.0 ± 0.1[f]	21.7 ± 2.9[e]	0.6 ± 0.1	0.3 ± 0.2[f]	0.1 ± 0.05	1.4 ± 0.2	15.4 ± 3.6
						HEART					
PC-C	18.9 ± 1.1	21.6 ± 1.5	8.8 ± 0.6	12.9 ± 2.4	0.3 ± 0.08	26.4 ± 2.3	0.3 ± 0.1	0.2 ± 0.07	0.2 ± 0.04	1.8 ± 0.2	7.0 ± 0.8
D	16.0 ± 1.8[e]	22.7 ± 1.7	7.1 ± 0.5[f]	23.9 ± 6.7[e]	0.6 ± 0.2[e]	19.1 ± 4.5[e]	0.2 ± 0.1	-	-	1.8 ± 0.4	7.7 ± 2.1
PI-C	3.1 ± 0.8	37.5 ± 2.7	4.8 ± 0.6	5.2 ± 1.1	0.6 ± 0.1	30.3 ± 2.2	-	0.7 ± 0.2	1.5 ± 0.8	2.3 ± 0.6	13.3 ± 1.7
D	2.7 ± 0.8	37.7 ± 1.7	3.8 ± 1.4	7.2 ± 3.1	1.4 ± 0.5[d]	31.5 ± 4.7	-	-	-	2.2 ± 0.6	12.2 ± 2.3
PE-C	9.7 ± 0.7	20.0 ± 0.9	7.0 ± 0.6	19.2 ± 1.2	0.2 ± 0.03	13.7 ± 1.3	0.3 ± 1.3	0.5 ± 0.07	0.5 ± 0.06	2.2 ± 0.2	23.8 ± 1.5
D	9.2 ± 1.1	20.2 ± 1.4	6.9 ± 0.5	18.5 ± 4.4	0.3 ± 0.05[f]	12.5 ± 1.2[a]	0.2 ± 0.03	0.4 ± 0.06	0.6 ± 0.07	2.6 ± 0.4	26.4 ± 2.8[a]
						RED BLOOD CELLS					
PC-C	46.0 ± 1.9	16.7 ± 0.8	9.1 ± 0.7	15.2 ± 1.1	0.6 ± 0.1	7.9 ± 1.5	0.2 ± 0.1	-	-	0.2 ± 0.2	1.2 ± 0.6
D	41.1 ± 2.7[d]	19.2 ± 0.7[f]	8.1 ± 0.7[a]	21.8 ± 1.5[f]	0.6 ± 0.1	5.7 ± 1.7[a]	0.2 ± 0.1	-	-	0.1 ± 0.1	1.0 ± 0.3
PI-C	7.4 ± 0.8	25.8 ± 1.2	6.6 ± 0.2	6.0 ± 0.4	0.6 ± 0.04	42.8 ± 1.7	1.2 ± 0.4	1.7 ± 0.2	-	1.2 ± 0.2	6.0 ± 0.3
D	8.6 ± 2.2	27.5 ± 2.0	6.2 ± 0.5	8.3 ± 1.7[a]	0.9 ± 0.1[c]	38.3 ± 4.5	1.2 ± 0.2	-	-	1.1 ± 0.3	5.8 ± 1.1
PE-C	13.6 ± 1.6	13.0 ± 0.9	14.9 ± 0.9	6.0 ± 0.5	-	32.7 ± 1.0	1.1 ± 0.2	2.2 ± 0.3	0.6 ± 0.2	5.6 ± 0.7	5.7 ± 1.4
D	13.2 ± 1.3	13.3 ± 1.4	15.2 ± 0.6	7.8 ± 0.8[e]	0.2 ± 0.05	31.2 ± 1.6	1.3 ± 0.1	1.7 ± 0.2[e]	0.4 ± 0.04	4.6 ± 2.4	6.8 ± 0.6
						KIDNEY					
PC-C	43.0 ± 3.8	11.2 ± 0.9	11.3 ± 0.6	15.0 ± 1.3	0.9 ± 0.2	12.8 ± 2.9	0.4 ± 0.2	-	-	0.3 ± 0.1	1.6 ± 0.5
D	30.1 ± 2.5[f]	15.9 ± 1.2[f]	11.4 ± 0.6	21.7 ± 2.9[f]	1.4 ± 2.9[f]	13.8 ± 2.8	0.4 ± 0.1	-	-	0.3 ± 0.1	2.7 ± 0.8[e]
PI-C	9.4 ± 1.2	30.4 ± 2.3	7.1 ± 0.9	5.4 ± 1.5	2.1 ± 0.3	39.8 ± 2.2	1.0 ± 0.3	0.6 ± 0.1	0.2 ± 0.2	0.7 ± 0.1	2.2 ± 0.4
D	7.6 ± 0.6[e]	32.5 ± 3.1	6.0 ± 0.7[c]	6.0 ± 0.7	3.1 ± 0.7[f]	40.3 ± 2.6	0.8 ± 0.2	0.5 ± 0.05	0.2 ± 0.1	0.6 ± 0.08	1.8 ± 0.3[b]
PE-C	10.2 ± 4.2	34.0 ± 6.5	6.2 ± 1.4	4.8 ± 1.8	1.8 ± 0.4	36.7 ± 2.5	1.0 ± 0.2	0.5 ± 0.2	-	0.7 ± 0.1	3.0 ± 1.5
D	6.5 ± 1.7[b]	39.0 ± 4.3	5.2 ± 1.6	5.5 ± 2.8	2.6 ± 1.0[a]	36.3 ± 2.3	0.8 ± 0.2	0.5 ± 0.1	-	0.6 ± 0.1	1.8 ± 0.4[a]
						TESTIS					
PC-C	38.9 ± 0.4	3.9 ± 0.3	14.1 ± 0.5	4.7 ± 0.5	1.7 ± 0.3	15.8 ± 0.7	-	1.0 ± 0.07	16.1 ± 0.8	0.8 ± 0.1	0.9 ± 0.1
D	38.3 ± 0.6	3.5 ± 0.3	14.2 ± 0.7	5.2 ± 0.7	2.1 ± 0.1[e]	14.6 ± 0.9[e]	-	0.9 ± 0.08	17.1 ± 0.9[d]	0.8 ± 0.1	0.9 ± 0.02
PI-C	28.7 ± 0.5	19.3 ± 0.9	5.2 ± 0.5	1.3 ± 0.1	0.6 ± 0.1	25.3 ± 3.0	-	1.5 ± 0.2	15.4 ± 0.6	0.9 ± 0.1	0.9 ± 0.02
D	28.3 ± 3.1	20.0 ± 1.9	5.1 ± 0.4	1.6 ± 0.2[a]	0.7 ± 0.1	24.9 ± 1.8	-	1.3 ± 0.1	16.1 ± 1.2	0.8 ± 0.1	1.1 ± 0.06[d]
PE-C	31.4 ± 1.2	6.4 ± 0.9	7.9 ± 0.5	4.9 ± 2.0	0.6 ± 0.2	20.2 ± 1.1	-	2.2 ± 0.2	21.5 ± 1.5	1.4 ± 0.2	0.6 ± 0.1
D	30.2 ± 1.3	6.7 ± 1.0	7.8 ± 0.7	4.6 ± 0.9	0.6 ± 0.08	19.7 ± 2.5	-	2.0 ± 0.2	22.7 ± 1.7	1.3 ± 0.2	0.6 ± 0.2

reduced and 18:0 increased in heart, kidney, adipose tissue and skin. Changes in oleic acid were also small, except in skin, where a dramatic fall took place. (b) Linoleic acid in PL was elevated in all tissues except the testis where it was unchanged and the skin where it was reduced. (c) 20:3n-6 In PL was elevated in every tissue. Its immediate metabolite, arachidonic acid, was reduced in every tissue but the skin and adrenal gland. Except for these last 2 tissues, the ratio of 20:3n-6 to 20:4n-6 was substantially above the controls. (d) Changes in the C22n-6 fatty acids were rather small but 22:4n-6 was down in every tissue in which it was present, and 22:5n-6 was sharply reduced in adipose tissue. (e) Levels of the n-3 acids were low and changes were small. However, in 20:5 n-3, the n-3 equivalent of arachidonic acid was consistently reduced. (f) The detailed changes shown in Table 2 generally show similar trends in all 3 phospholipid fractions measured. The 18:2n-6 elevations were consistently greater in PC than PI+PS. The 20:3n-6 elevations, however, were consistently as large in PI+PS as in PC. Changes in 20:4n-6 were substantial in the liver in both PC and PI+PS, but the heart showed change only in PC. Changes in 20:4n-6 in other tissues were small. The kidney showed changes in all 3 fractions in 22:6n-3, with a rise in PC and fall in PI+PS and PE. (g) The skin was remarkable in consistently showing changes opposite from other tissues.

DISCUSSION

In general, our observations are similar to those reported by others (1-9) but are more detailed and cover a larger number of tissues. The observed changes in n-6 and n-3 essential fatty acids (EFA) in the PL could be related either to changed pattern of incorporation from a free fatty acid pool of unchanged composition, or to unchanged incorporation from a fatty acid pool where composition has changed or to a mixture of both mechanisms. Substantial evidence already exists that desaturation of EFA by both the Δ6 and Δ5 enzyme is reduced in diabetes (1-6), suggesting that the pool of fatty acids available for incorporation into PL has changed. On the other hand, changes in incorporation may also occur as suggested by the levels of 22:6n-3 in individual PL in the kidney, where diabetes produced changes in opposite directions in the different fractions. We suspect that both mechanisms may be involved but that the changes in desaturation already demonstrated by others are the more important factors.

The very consistent elevation of 18:2n-6 has been repeatedly reported by others and is consistent with, though not proof of, inhibition of Δ6 desaturation. The 20:3n-6 levels have not usually been reported in previous studies, except by Holman et al. (9), whose observation are similar. Our findings of elevated 20:3n-6 and reduced 20:4n-6 are consistent with reduced Δ5 desaturation activity, as has been reported by direct microsomal studies (14). The low 20:5n-3 levels are also consistent with this.

The fatty acid patterns show rather large differences from tissue to tissue, which are probably explained both by different mechanisms of fatty acid incorporation into PL and also by different patterns of desaturation and elongation. On the whole, however, the change produced by diabetes was similar in direction in all tissues. The one major exception to this was the skin in which 18:2n-6 was strikingly reduced and 20:4n-6 significantly increased. Thus, generalizing about the effects of diabetes from tissue where measurements of fatty acid levels have been made to one where they have not been made is not possible. Each tissue appears to have a specific mechanism for handling fatty acids and, in some cases, regulating factors, e.g., hormones, may have opposite effects in different tissue.

REFERENCES

1. Faas, F.H., and Carter, W.J. (1980) Lipids 15, 953-961.
2. Faas, F.H., and Carter, W.J. (1983) Lipids 28, 339-342.
3. Mercuri, O., Peluffo, R.O., and Brenner, R.R. (1966) Biochim. Biophys. Acta 116, 409-411.
4. Brenner, R.R. (1974) Mol. Cell Biochem. 3, 41-52.
5. Friedman, N., Gellhorn, A., and Benjamin, W. (1966) Israel J. Med. Sci. 2, 677-682.
6. Poisson, J.P., Lemarchal, P., Blond, J.P., Lecerf, J., and Mendy, F. (1978) Diabetes Metab. 4, 39-45.
7. Brenner, R.R., Peluffo, P.O., Mercuri, O., and Restelli, M.A. (1968) Am. J. Physiol. 215, 63-70.
8. Clark, D.L., Hamel, F.G., and Queener, S.F. (1983) Lipids 18, 696-705.
9. Holman, R.T., Johnson, S.B., Gerrard, J.M., Mauer, S.M., Kupcho-Sandberg, S., and Brown, D.M. (1983) Proc. Natl. Acad. Sci. USA 80, 2375-2379.
10. Huang, Y.S., Martineau, A., Falardeau, P., and Davignon, J. (1983) Lipids 18, 412-422.
11. Skipski, V.P., Peterson, R.F., and Barclay, M. (1964) Biochem. J. 90, 374-378.
12. Morrison, W.R., and Smith, L.M. (1964) J. Lipid Rds. 5, 600-608.
13. Manku, M.S., Horrobin, D.F., Huang, Y.S., and Morse, N. (1983) Lipids 18, 906-908.
14. Brenner, R.R. (1971) Lipids 6, 567-575.

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Inhibition of Human Lecithin Cholesterol Acyltransferase by Monoterpenes

ROBERT V. COONEY*, JEFFREY NEMHAUSER and **ROBERT J. MORIN,** *Department of Pathology, Harbor-UCLA Medical Center, Torrance, CA 90509*

ABSTRACT

The lecithin-cholesterol acyltransferase activity of human plasma was found to be inhibited by Rowachol, a proprietary mixture of pure monoterpenes. Menthol, the major ingredient in Rowachol (32%), and a number of other monoterpenes were found to inhibit the enzyme independently. Concentrations of monoterpenes required to achieve 50% inhibition were of the same order of magnitude as the cholesterol concentration present in the reaction mixture.
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INTRODUCTION

The incidence of coronary heart disease has been shown to be inversely correlated with serum high density lipoprotein (HDL) cholesterol concentration in man (1-3). HDL may function in conjunction with the enzyme lecithin–cholesterol acyltransferase to bring about the efflux of cholesterol from peripheral tissues to sites of catabolism (4,5). In vitro evidence for such a transport role has been obtained by Stein et al. (6) using human skin fibroblasts. Lecithin-cholesterol acyltransferase (LCAT) is thought to assist in this process by increasing the capacity of HDL particles for cholesterol storage by shifting the equilibrium between membrane and plasma cholesterol (7,8).

Rowachol, a proprietary choleretic preparation composed of menthol (32% w/w), pinene (17% w/w), menthone (6% w/w), borneol (5% w/w), camphene (5% w/w) and cineole (2% w/w) in olive oil, has been shown to elevate serum HDL-cholesterol concentrations in humans (9). In addition, Benko et al. (10) showed that monoterpenes could prevent the formation of atherosclerotic plaques in cholesterol-fed rabbits. Middleton et al. (11) found that Rowachol reduced the in vivo activity of S-3-Hydroxy-3 methylglutaryl-CoA reductase, an enzyme involved with cholesterol biosynthesis, but the monoterpenes did not inhibit the enzyme directly in vitro. Because lecithin-cholesterol acyltransferase has been implicated in the process of cholesterol transport by HDL and monoterpenes are known to increase serum HDL-cholesterol, our laboratory undertook an investigation into the effects of Rowachol and monoterpenes on human plasma LCAT activity. The present paper describes the inhibition of LCAT by a number of pure monoterpenes as well as Rowachol.

MATERIALS AND METHODS

Rowachol was generously provided by ROWA Limited Pharmaceuticals of Bantry Co., Cork, Ireland. Menthol camphor, menthone, cineole, borneol and digitonin were purchased from Sigma Chemical Company, St. Louis, MO. [7n-^{3}H]Cholesterol was obtained from Amersham Corp., Arlington Heights, IL. All other chemicals were reagent grade.

Enzyme assays were carried out according to the method of Stokke and Norum (12). Each sample contained 200 µl of freshly pooled human plasma, 40 µl of 10 mM Ellman reagent, 50 µl of ^{3}H-substrate and 10 µl of either olive oil or olive oil plus inhibitor. The mixture was preincubated for 2 hr at 37 C to allow for equilibration of substrate, label and inhibitor. The reaction was started by adding 40 µl of 0.1 M mercaptoethanol. The samples were incubated for 3 hr after which time the conversion of ^{3}H-cholesterol to cholesterol ester was determined using the digitonin precipitation method of Piran and Morin (13). Aliquots of 100 µl were removed from the supernatant after precipitation and counted in 3.0 ml of ACS liquid scintillation counting fluid in a Packard Tricarb scintillation counter. Values reported represent the means of duplicate determinations repeated 4 times.

RESULTS AND DISCUSSION

Rowachol was found to inhibit LCAT activity at a variety of concentrations tested (Table 1). Complete inhibition occurred when 2 µl or more of Rowachol were used in our assay system. Because Rowachol is a mixture of chemicals, giving a molar concentration of Rowachol per se is not possible; however, 2 µl would correspond to a 20 mM menthol concentration in the assay.

TABLE 1

Inhibition of Lecithin-Cholesterol Acyltransferase by Rowachol

μl Rowachol	Menthol concentration (mM)	cpm[a]	Inhibition (%)
0	0	1954 ± 66	0
0.05	0.05	2010 ± 50	0
0.1	1	2114 ± 144	0
0.2	2	1860 ± 40	4.8
0.5	5	1222 ± 52	37.5
1.0	10	576 ± 15	70.5
2.0	20	0	100
5.0	50	0	100

[a]Mean cpm above blank ± standard deviation (n = 4).

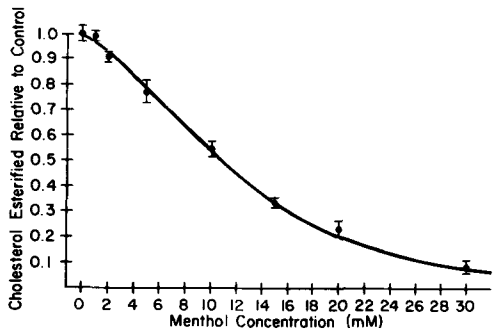

FIG. 1. Effect of methol concentration on cholesterol esterification.

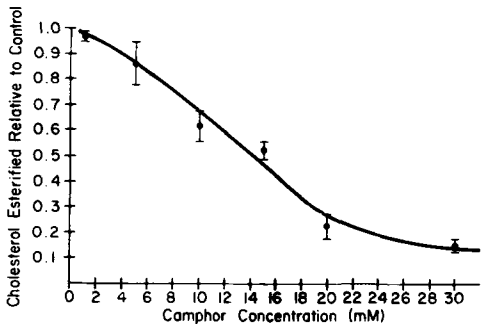

FIG. 2. Effect of camphor concentration on cholesterol esterification.

Figures 1 and 2 show the effects on LCAT of menthol and camphor, respectively. A menthol concentration of ca. 11 mM resulted in 50% inhibition of LCAT, whereas 14 mM camphor was required to achieve the same degree of inhibition. Menthone also produced a similar concentration-dependent inhibition of LCAT. We were unable to test the effect of borneol on LCAT because of its limited solubility in our assay system. Cineole appeared to inhibit LCAT to some extent; however, results were very inconsistent with this compound and not reproducible because of the wide scattering of data.

All of the monoterpenes tested, therefore, inhibited LCAT to varying extents. These results are consistent with the observed inhibition of LCAT by Rowachol, which is greater than that predicted on the basis of its menthol content.

The monoterpenes may be acting as competitive inhibitors to LCAT because the concentration of monoterpene required to achieve 50% inhibition is of the same order of magnitude as the cholesterol concentration in the enzyme assay (ca. 6 mM). additional experiments with highly purified enzyme and substrates prepared with synthetic liposomes may shed further light on the specific mechanism involved in inhibition and the relative affinities of LCAT for cholesterol and various monoterpenes. An interesting question is raised in this regard as to whether the enzyme may be esterifying a fatty acid to menthol or competing for the active site without serving as a substrate. LCAT is known to use H_2O as an acyl acceptor as well as some other alcohols (14). Work by Morin and Ihrig (15) on the formation of palmitate esters of monohydric short-chain alcohols by swine arterial subcellular fractions suggests that some esterifying enzymes may not be highly specific, lending some credibility to the former hypothesis. If menthol is esterified by LCAT, menthone and camphor might represent longer lasting inhibitors in vivo because of their lack of a hydroxyl group.

The finding that Rowachol and menthol inhibit lecithin-cholesterol acyltransferase is particularly intriguing in light of the prevailing

view that LCAT may function to increase plasma HDL-cholesterol (16). One theory currently holds that LCAT functions with HDL in a reverse cholesterol transport pathway (17). Such a theory predicts a direct correlation between LCAT activity and serum HDL-cholesterol levels. Our finding that monoterpenes inhibit LCAT, while increasing serum HDL-cholesterol (9), suggests a different and more complex relation between HDL, LCAT and cholesterol removal than previously thought. Interestingly, some clinical support for our observations is provided by Soloff and Varma (18) who reported an inverse relation between LCAT activity and serum HDL-cholesterol concentration in humans.

The significance and relationship of our in vitro findings to the in vivo action of monoterpenes remains to be determined. In the early work of Benko et al. (10), an equimolar quantity of monoterpenes relative to administered cholesterol, was required to prevent the formation of atherosclerotic plaques. Such a dose produced a plasma cholesterol concentration of ca. 25 mM. Because monoterpenes were administered at the same dose level as cholesterol, millimolar levels of the former may have been achieved. This report provides the first evidence for a direct effect of monoterpenes on the activity of an enzyme involved in cholesterol metabolism. The relation between this effect in vitro and the observed elevation of plasma HDL-cholesterol and the prevention of atherosclerosis in vivo by monoterpenes deserves further study in vivo. Additionally, other similar compounds need to be screened for their ability to inhibit LCAT at lower concentrations.

The method used in the current study (12) is well suited for a comparative study of intrinsic plasma LCAT activity in normal vs monoterpene-treated patients. Such a study would provide information as to whether or not LCAT inhibition can be achieved in vivo as well. Depending on the $t_{1/2}$ for menthol in plasma, monoterpene concentrations in plasma in the inhibitory range could be achieved. Elder et al. showed that menthol, when given orally (13 mg/kg), stimulated galactose oxidation in vivo; millimolar levels are required to achieve the same effect on the enzyme system in vitro (19). Further research into the mechanisms of action of the monoterpenes may also contribute to our understanding of the processes involved in cholesterol transport and metabolism.

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REFERENCES

1. Gordon, T., Castelli, W.P., Hjortland, M.C., Kannel, W.B., and Dawber, T.R. (1977) Am. J. Med. 62, 707-714.
2. Miller, N.E., Forde, O.H., Thelle, D.S., and Mjos, O.D. (1977) Lancet 1, 965-968, 985.
3. Goldbourt, U., and Medailie, J.H. (1979) Am. J. Epidemiol. 109, 296-308.
4. d'Hollander, F., and Chevallier, F. (1972) Biochim. Biophys. Acta 260, 110-132.
5. Glomset, J.A. (1970) Am. J. Clin. Nutr. 23, 1129-1136.
6. Stein, O., Vanderhoek, J., Friedman, G., and Stein, (1976) Biochim. Biophys. Acta 450, 367-378.
7. Murphy, J.R. (1962) J. Lab. Clin. Med. 60, 86-109.
8. Glomset, J.A., Norum, K.R., and King, W. (1970) 49, 1827-1837.
9. Bell, G.D., Bradshaw, J.P., Burgess, A., Ellis, W., Hatton, J., Middleton, A., Middleton, B., Orchard, T., and White, D.A. (1980) Atherosclerosis 36, 47-54.
10. Benko, S., Macher, A., Szarvas, F., and Tiboldi, T. (1961) Nature 190, 731-732.
11. Middleton, A., Middleton, B., White, D.A., and Bell, G.D. (1979) Biochem. Soc. Trans. 7, 407-408.
12. Stokke, K.T., and Norum, R.J. (1971) Scand. J. Clin. Lab. Invest. 27, 21-27.
13. Piran, U., and Morin, R.J. (1979) J. Lipid Res. 20, 1040-1043.
14. Aron, L., Jones, S., and Fielding, C.J. (1979) J. Biol. Chem. 253, 7220-7226.
15. Morin, R.J., and Ihrig, T.J. (1974) Lipids 9, 86-90.
16. Wallentin, L., and Moberg, B. (1982) Atherosclerosis 41, 155-165.
17. Glomset J.A. (1979) Biochem. Pharmacol. 15, 41-66.
18. Soloff, L.A., and Varma, K.G. (1978) Scand. J. Clin. Lab. Invest. 38, suppl. 150, 72-76.
19. Elder, T.D., Segal, S., Maxwell, E.S., and Topper, Y.J. (1960) Science 132, 225-226.

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Volatiles from Thermal Decomposition of Isomeric Methyl (12*S*,13*S*)-(*E*)-12,13-epoxy-9-hydroperoxy-10-octadecenoates

H.W. GARDNER and **E. SELKE**, *Northern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Peoria, IL 61604*

ABSTRACT

Two epimers of methyl (12S,13S)-(E)-12,13-epoxy-9-hydroperoxy-10-octadecenoate were isolated after esterification of a mixture of fatty acids obtained from decomposition of (13S)-(9Z,11E)-13-hydroperoxy-9,11-octadecadienoic acid by an Fe^{++}-cysteine catalyst. These epimeric epoxyhydroperoxyoctadecenoates were decomposed by heat (210 C) in the injection port of a gas chromatograph, and the cleavage fragments were subsequently separated by gas chromatography (GC) and identified by mass spectrometry (MS). Among the scission products obtained, the most prominent in the GC peak profile were methyl octanoate and methyl 9-oxononanoate. Other peaks were identified as pentane, 1-pentanol, hexanal, 2-heptanone, 2-pentylfuran, methyl heptanoate, 2-octenal, 4,5-epoxy-2-decenal, methyl 8-(2-furyl)-octanoate, 11-oxo-9-undecenoate and methyl 13-oxo-9,11-tridecadienoate. In addition, 3,4-epoxynonanal, methyl 8-oxooctanoate, 3-hydroxy-2-pentyl-2,3-dihydrofuran and methyl 10-oxodecanoate were tentatively identified. Except for the furan compounds, the formation of the fragmentation products could be explained by conventional free-radical scission mechanisms.
Lipids 19:375-380, 1984.

INTRODUCTION

Homolysis of the hydroperoxide group leads to chain cleavage of fatty hydroperoxides by the well-known β-scission of the oxy radical (1). This mechanism has satisfactorily explained the formation of a majority of volatiles from the decomposition of autoxidized lipids and pure fatty hydroperoxides.

The isolation of 4,5-epoxy-2-heptenal from the Cu/α-tocopherol-induced autoxidation of either butterfat or cod liver oil (2) stimulated interest in the origin of epoxide carbonyls. Subsequently, the same carbonyl was identified as a thermal decomposition product of methyl linolenate hydroperoxides (3). According to Swoboda and Peers (2), 4,5-epoxy-2-heptenal could originate from 15,16-epoxy-12-hydroperoxy-9,13-octadecadienoic acid produced by further oxidation of the 16-hydroperoxide of linolenic acid. A similar carbonyl, 4,5-epoxy-2-decenal, was tentatively identified by Selke et al. (4) as a decomposition product of autoxidized trilinolein. They postulated that 4,5-epoxy-2-decenal could arise from 12,13-epoxy-9-hydroperoxy-10-octadecenoate, which had been isolated previously (5) as an oxidation product of the 13-hydroperoxide of linoleate. Recently, methyl 12,13-epoxy-9-hydroperoxy-10-octadecenoate also has been isolated from autoxidized methyl linoleate (6).

To substantiate the origin of epoxide aldehydes from epoxyhydroperoxy fatty acids or their esters, we isolated isomeric methyl 12,-13-epoxy-9-hydroperoxy-10-octadecenoates for thermal decomposition studies.

METHODS

Preparation of Hydroperoxides

(13S)-(9Z,11E)-13-hydroperoxy-9,11-octadecadienoic acid was obtained by soy lipoxygenase oxidation of linoleic acid (NuChek Prep., Elysian, MN) followed by isolation with column chromatography (7). The purity of the isolate was analyzed by high performance liquid chromatography (HPLC) and found to be 99%. The HPLC system and conditions were the same as reported below for the separation of isomeric methyl epoxyhydroperoxyoctadecenoates.

To prepare 12,13-epoxy-9-hydroperoxy-10-octadecenoic acid, the 13-hydroperoxy-9,11-octadecadienoic acid (162 mg) was reacted in a solution containing $FeCl_3$ and cysteine as described previously (5,8). Because the epoxyhydroperoxyoctadecenoic acids also were decomposed by this reagent, care had to be exercised to terminate the reaction at a time when maximum yields could be realized (as assessed by TLC). Thus, the solution was treated with pure O_2 at 25 C for 10 min, and terminated with the addition of 130 ml 1 mM Na_2EDTA (aqueous) and 325 ml $CHCl_3$. The $CHCl_3$ layer containing the fatty acid products was washed twice with 150 ml H_2O. The recovered fatty

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acids were esterified with diazomethane before chromatographic separation.

Chromatographic Methods

Separation of methyl epoxyhydroperoxyoctadecenoates from other fatty esters was accomplished by chromatography of the esters on a column (i.d. 2.5 cm) packed with 50 g SilicAR CC7 (Mallinckrodt). Step-by-step elution was performed with 0.25 l of 10% (by vol) diethyl ether in hexane followed by ether in hexane in the proportions of 0.25 l, 20%; 0.30 l, 25%; 0.30 l, 30%. The isomeric methyl epoxyhydroperoxyoctadecenoates (16.7 mg) eluted between 0.62-0.83 l as determined by TLC (Silica Gel G; hexane/diethyl ether [7.3, v/v]) of a small portion of the fractions and spraying the plates with peroxide-specific ferrous thiocyanate solution (9).

The esters isolated by column chromatography were further separated by HPLC (9.4 x 250 mm Partisil M-9 column, Whatman) using 6% acetone in hexane at a flow rate of 3 ml/min. A Perkin Elmer LC-75 spectrophotometric detector set at 206 nm monitored the eluants. Isomeric methyl epoxyhydroperoxyoctadecenoates separated as nearly equal peaks and were designated as isomers A and B, eluting at 65 min and 73 min, respectively. Isomer A was adequately purified after the initial HPLC separation, but B required refractionation by HPLC to remove small amounts of A and a slower moving component. Compounds A and B were examined by proton nuclear magnetic resonance spectrometry (^{1}H-NMR) using a Bruker WH-90 operating at 90 MHz. For ^{1}H-NMR the samples were dissolved in $CDCl_3$, with 1% tetramethylsilane serving as an internal standard.

Gas Chromatography-Mass Spectrometry

Between 0.5-0.7 mg of epoxyhydroperoxides A and B were dissolved in 6-8 μl CS_2 and inserted into the injection port (210 C) of a Bendix 2600 series gas chromatograph. Thermal decomposition products of epoxyhydroperoxides A and B were separated on a glass column (4.9 m x 4 mm) packed with 3% JXR on gas chromosorb Q. Initially, the He carrier flow was ca. 5 ml/min just before and 1 min after sample injection, and then the flow was increased to ca. 36 ml/min. The column temperature was programmed from 0 C to 275 C at 2 C/min. As the volatiles eluted from the column, they were monitored with jet separator by a Nuclide MS (12-90 double focussing, magnetic scanning) set to scan from m/z 10 to m/z 450 at 9 sec intervals. Output from the MS was stored by computer for later processing. Volatiles were identified by matching their MS to those in a 35,000 standard library and confirmed by GC retention data. The computerized GC-MS system has been described previously (10).

RESULTS AND DISCUSSION

Methyl 12,13-epoxy-9-hydroperoxy-10-octadecenoate, used to produce volatiles, has been characterized before (5), and the same method of preparation was used in this study. However, in the previous work this compound was isolated by methods (column chromatography and TLC) that gave no apparent separation of isomers. In this study, HPLC afforded isolation of 2 isomers, A and B. ^{1}H-NMR (90 MHz) of each revealed insignificant differences in spectral data between the pair, and the spectrum of each isomer was comparable to that reported previously for the apparent isomeric mixture (5), i.e., for both isomers the epoxide and double bond were both *trans*. In the previous study the hydroperoxide (OOH) absorption was not reported, and we can now report this absorption as a singlet at δ7.83 for A and at δ7.85 for B.

On the basis of previous work (5,11), the pair was assigned a 12S,13S chirality (based on the 13S-hydroperoxide precursor and the *trans* configuration of the epoxide). The chirality at C 9 (9S vs 9R) was not defined, thus A and B may be the diastereomeric pair, methyl (9S,12S,13S)-(E)-12,13-epoxy-9-hydroperoxy-10-octadecenoate and methyl (9R,12S,13S)-(E)-12,13-epoxy-9-hydroperoxy-10-octadecenoate. An analogous pair, methyl (9S,12S,13S)-(E)-12,13-epoxy-9-hydroxy-10-octadecenoate and methyl (9R,12S,13S)-(E)-12,13-epoxy-9-hydroxy-10-octadecenoate, also originated from the 13S-hydroperoxide of linoleic acid; this pair was separated by the same HPLC technique (12). These investigators (12) also found that their pair of diastereomers could not be distinquished by 90 MHz ^{1}H-NMR. In future studies we will attempt to define the specific chirality of the A and B isomers, as well as other epoxyhydroperoxy isomers not reported here.

The thermal (210 C) decomposition products of epoxyhydroperoxide A are shown in Figure 1. Although the GC profile for epoxyhydroperoxide B is not shown, this isomer gave similar volatiles, both qualitatively and by peak size (Table 1). Methyl octanoate and methyl 9-oxononanoate were the main volatiles (>13%) from thermal decomposition. Other significant cleavage fragments (>1.7%) were pentane,

hexanal, 2-pentylfuran, methyl 8-(2-furyl)-octanoate, methyl 11-oxo-9-undecenoate, methyl 13-oxo-9,11-tridecadienoate, 4,5-epoxy-2-decenal (the expected volatile) and 3 tentatively identified peaks (3,4-epoxynonanal, methyl 8-oxooctanoate and 3-hydroxy-2-pentyl-2,3-dihydrofuran). Minor volatiles (<1.7%) were 1-pentanol, 2-heptanone, methyl heptanoate and 2-octenal, and 2 of these minor peaks were tentatively identified as heptanal and methyl 10-oxodecanoate. The identities of pentane, 1-pentanol, hexanal, 2-heptanone, 2-pentylfuran, methyl heptanoate, 2-octenal, methyl octanoate, methyl 9-oxononanoate and methyl 11-oxo-9-undecenoate were easily ascertained from their elution temperature and from comparison of MS data with library spectra. The cleavage fragment, 4,5-epoxy-2-decenal, afforded MS and GC data comparable to those published by Selke et al. (4), except that we obtained additional ions as follows: m/z (relative percentage, ion structure), 152 (2, M^+-O), 150 (2, M^+-H_2O), 127 (3), 109 (7), 107 (8), 95 (9), 81 (40), 57 (33) and 28 (30). The peaks identified as methyl 8-(2-furyl)-octanoate and methyl 13-oxo-9,11-tridecadienoate gave MS that compared favorably with those recently published (13). A few additional ions of moderate intensity were observed in this study for methyl 8-(2-furyl)-octanoate [m/z (rel. percentage), 59 (11), 55 (17), 53 (22) and 41 (19)] and for methyl 13-oxo-9,11-tridecadienoate [m/z (rel. percentage), 178 (3), 149 (22), 121 (6), 79 (27), 67 (33) and 41 (48)].

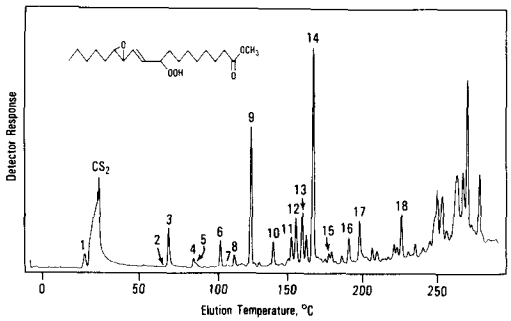


FIG. 1. Gas chromatography of thermally induced (210 C) products from methyl 12,13-epoxy-9-hydroperoxy-10-octadecenoate (isomer A). Peak response was by a flame ionization detector. Identification of volatiles by peak number is keyed to Table 1.

TABLE 1

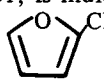
Relative Gas Chromatographic Peak Areas of Volatiles[a] from Thermal Decomposition of Two Isomers (A and B) of Methyl 12,13-Epoxy-9-hydroperoxy-10-octadecenoate

Volatile compounds	Peak number[b]	Relative percentage A	Relative percentage B
Pentane	1	1.9	2.7
1-Pentanol	2	0.1	0.2
Hexanal	3	4.3	4.8
2-Heptanone	4	0.8	0.9
Heptanal[c]	5	0.6	0.3
2-Pentylfuran	6	2.4	5.6
Methyl heptanoate	7	0.3	0.1
2-Octenal	8	1.3	1.0
Methyl octanoate	9	13	14
3,4-Epoxynonanal[c]	10	2.6	6.5
Methyl 8-oxooctanoate[c]	11	2.7	1.7
3-Hydroxy-2-pentyl-2,3-dihydrofuran[c]	12	5.2	5.1
4,5-Epoxy-2-decenal	13	5.6	2.7
Methyl 9-oxononanoate	14	23	31
Methyl 10-oxodecanoate[c]	15	1.2	1.0
Methyl 8-(2-furyl)-octanoate	16	3.2	3.8
Methyl 11-oxo-9-undecenoate	17	4.7	3.3
Methyl 13-oxo-9,11-tridecadienoate	18	4.4	2.1
Unidentified		23	13

[a]The relative peak areas are determined from flame ionization detection, and are reported as percentage of the total volatiles eluting from the pentane peak to methyl 13-oxo-9,11-tridecadienoate. The solvent peak (CS_2) and compounds with retention longer than methyl 13-oxo-9,11-tridecadienoate were excluded from the calculations. The data are uncorrected for response factors, thus the tabulated values probably deviate somewhat from the absolute.

[b]For peak numbers see Figure 1.

[c]Tentative identification.

For various reasons, 6 volatiles of moderate peak size could not be identified with confidence; however, the data permitted tentative assignments for 5 of these. Peak 5 was thought to be heptanal from its expected elution temperature, but its MS was not definite enough to allow identification because of the small size of the sample. Methyl 10-oxodecanoate was indicated for peak 15 from a library match of its MS and its elution temperature; however, the GC characteristics of a standard have not been tested with our equipment. Peak 10 was presumed to be 3,4-epoxynonanal on the basis of its elution characteristics and the following MS data: m/z (rel. percentage, ion structure), 138 (2, M^+-H_2O), 128 (3, M^+-CO), 127 (2, M^+-CHO), 113 (3, M^+-CH_2CHO), 110 [2, M^+-(H_2O + CO)], 100 (18), 99 [29, $CH_3(CH_2)_4CHO^+$], 85 [51, M^+-$CH_3(CH_2)_4$], 72 (38), 71 [42, CH_3-$(CH_2)_4^+$], 58 (13), 57 (24), 55 (20), 43 (100), 41 (28), 29 (53), and 27 (27). The MS from peak 12 was similar to that obtained with peak 10, except for 2 additional ions: m/z (rel. percentage), 109 (9) and 81 (27). This volatile cannot be a homolog of 10 because its GC retention is ca. 1 equivalent chain length (ECL) larger. A plausible structure would be 3-hydroxy-2-pentyl-2,3-dihydrofuran for the following reasons: (a) more polar functionality (hydroxyl plus dihydrofuran) probably would cause the higher retention temperature compared with 3,4-epoxynonanal; (b) this compound has a molecular weight identical with 3,4-epoxynonanal, as well as a similar predicted pattern of MS fragment ions; (c) the additional diagnostic ion, m/z 81, is indicative of 2-alkyl substituted furans ( , 100% rel. intensity for 2-pentylfuran), and could be derived from ring dehydration. Methyl 8-oxooctanoate was indicated for peak 11 by its retention temperature and the following MS: m/z (rel. percentage, ion structure), 144 (6, M^+-CO), 141 (6, M^+-CH_3O), 129 (35), 101 (15), 97 (40), 95 (25), 87 (100), 81 (61), 74 (91), 69 (61), 59 (43), 55 (77), 43 (48), 41 (98), 29 (50), and 28 (77). The moderately sized peak migrating between peaks 13 and 14 could not be identified, but it probably was a methyl ester as surmised by the intense MS ions at m/z 59, 74 and 87.

Other decomposition products, eluting later than ca. 245 C, were thought to be C 18 fatty esters that did not undergo carbon-carbon cleavage. These components had apparent M^+ ions ranging between m/z 292-322. The large peak eluting at ca. 268 C resulted in a spectrum typical of either methyl 13-oxo-9,11-octadecadienoate, methyl 9-oxo-10,12-octadecadienoate or a mixture of both: m/z 308 (20%, M^+), 277 (6%, M^+-CH_3O), 252 [4%, $^+CH_2$=COH-(CH=CH)$_2$(CH_2)$_7$$COOCH_3$)], 237 [18%, M^+-$CH_3(CH_2)_4$], 187 (8%), 179 (10%), 177 (23%), 166 [12%, $CH_3(CH_2)_4(CH=CH)_2COH=CH_2^+$)], 159 (12%), 151 (100%, M^+-$(CH_2)_7COOCH_3$], 135 (20%), 121 (11%), 109 (22%), 107 (28%), 99 (37%), 95 (68%), 81 (92%), 67 (50%), 55 (63%), 43 (60%) and 41 (48%).

In the production of volatiles from epoxyhydroperoxides A and B, we used the heat of the GC injector port to induce molecular decomposition. This technique has been used extensively by others (e.g., 3,13-15) to decompose a variety of fatty hydroperoxides. The method is believed to result in a cascade of homolytic reactions that ultimately lead to β-scission of radicals and concomitant chain cleavage. Radical decomposition of epoxyhydroperoxides A and B predicts events leading to homolysis of the 9-hydroperoxide into an oxy radical. β-Scission of the oxy radical would fragment the carbon chain as indicated in Figure 2. Scission at C 8,9 would result in methyl octanoate and 4,5-epoxy-2-decenal, which were observed in this study. Similarly, the production of methyl 9-oxononanoate could be attributed to C 9,10 radical scission. However, the comparatively higher heat of formation of the vinyl radical involved in C 9,10 cleavage should disfavor this reaction. Although it could be argued (e.g., 1, 3, 15) that the compound tentatively identified as 3,4-epoxynonanal arose from reaction of the vinyl radical (Fig. 2) with a hydroxyl radical, 3,4-epoxynonanal as well as methyl 9-oxononanoate could also form via a heterolytic pathway (1,16), as shown in Figure 3. Until experiments are designed to define this cleavage between a

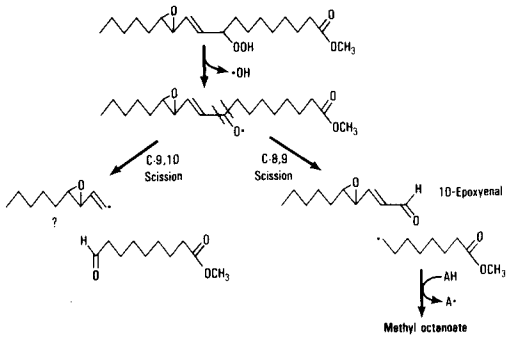


FIG. 2. Proposed pathways to volatiles from methyl 12,13-epoxy-9-hydroperoxy-10-octadecenoate. The question mark denotes a vinyl radical that is energetically unfavorable.

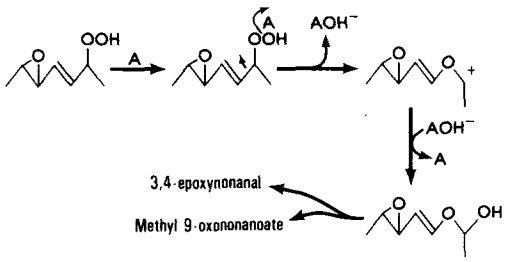


FIG. 3. Alternative heterolytic pathway to 3,4-epoxynonanal and methyl 9-oxononanoate. Structures are abbreviated to show only C 8 through C 4. An electron acceptor (A) could be a protic acid or a Lewis acid.

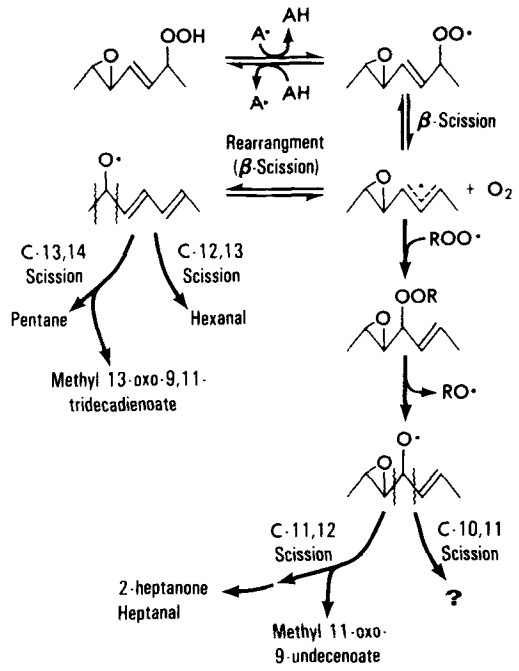


FIG. 4. Proposed pathways to pentane, hexanal, methyl 13-oxo-9,11-tridecadienoate, methyl 11-oxo-9-undecenoate, heptanal and 2-heptanone from methyl 12,13-epoxy-9-hydroperoxy-10-octadecenoate. Structures are abbreviated to show only C 8 through C 4.

hydroperoxy carbon and a double bond, the putative radical β-scission of the C 9,10 bond adequately explains the formation of methyl 9-oxononanoate in this study and also explains many cleavage fragments of other hydroperoxides (1). Thus, the β-scission model shown in Figure 2 could account for 3 and perhaps 4 of the volatiles, of which 2 were the most abundant. Comparing the volatile profile of Figure 1 with the one obtained with the thermal decomposition of methyl 9-hydroperoxy-10,12-octadecadienoate (17) is interesting. Except for the absence of 2,4-decadienals in this study, the major volatiles were comparable.

The appearance of small but significant amounts of pentane, hexanal and methyl 13-oxo-9,11-tridecadienoate was reminiscent of the volatiles obtained from thermal decomposition of methyl 13-hydroperoxy-9,11-octadecadienoate (16). Their origin can be readily explained by an equilibrium involving a variety of β-scission reactions and a rearrangement of a radical (Fig. 4). The β-scission of O_2 from a peroxy radical, demonstrated by Chan et al. (18) and others (19), makes the formation of an epoxyallylic radical from A and B appear feasible. The demonstrated rearrangement of propylene oxide radical (CH_2—CH-$CH_2^{\bullet}$) to propylene oxy radical (CH_2—CH=CH_2) (20) would make the rearrangement of the 12,13-epoxyallylic radical to 13-oxydiene radical seem plausible. β-Scission of the 13-oxydiene radical would then offer a postulated route to pentane, hexanal and methyl 13-oxo-9,11-tridecadienoate.

Figure 4 also shows a possible route to methyl 11-oxo-9-undecenoate, heptanal and 2-heptanone. An epoxyallylic radical derived from the epoxyhydroperoxide is proposed to react with alkyl peroxy radical generated thermally from a second molecule of epoxyhydroperoxide. Those peroxy radicals that combine with C 11 of the allylic radical, instead of C 9, would lead to production of an 11-oxy radical. β-Scission of the 11-oxy radical would lead directly to methyl 11-oxo-9-undecenoate, but the formation of heptanal and 2-heptanone would require additional transformations.

Appreciable quantities of both 2-pentylfuran and methyl 8-(2-furyl)-octanoate were found (Fig. 1). Although a number of mechanisms for furan formation have been suggested in a recent review (1) and by Sessa and Plattner (21), none of these would seem to apply directly to this study. The volatile tentatively identified as 3,4-epoxynonanal could lead to 2-pentylfuran by a reaction analogous to one used to synthesize furanoid fatty esters from epoxyoxo fatty acids by intramolecular attack of the oxo group on the epoxide (22). When this reaction was applied to 3,4-epoxynonanal, intramolecular nucleophilic attack of the enol form of the aldehyde on the C 4 epoxide would predict formation of 3-hydroxy-2-pentyl-2,3-dihydrofuran, a volatile tentatively identified as peak 12 in this study. Elimination of H_2O from this

compound would afford 2-pentylfuran. However, the analogous precursors for the formation of methyl 8-(2-furyl)-octanoate were not observed, thus the proposed pathway probably is not the only one to be considered for the production of furan volatiles.

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REFERENCES

1. Frankel, E.N. (1983) Prog. Lipid Res. 22, 1-33.
2. Swoboda, P.A.T., and Peers, K.E. (1978) J. Sci. Food Agric. 29, 803-807.
3. Frankel, E.N., Neff, W.E., and Selke, E. (1981) Lipids 16, 279-285.
4. Selke, E., Rohwedder, W.K., and Dutton, H.J. (1980) J. Am. Oil Chem. Soc. 57, 25-30.
5. Gardner, H.W., Weisleder, D., and Kleiman, R. (1978) Lipids 13, 246-252.
6. Imagawa, T., Kasai, S., Matsui, K., and Nakamura, T. (1982) J. Biochem. 92, 1109-1121.
7. Gardner, H.W. (1975) Lipids 10, 248-252.
8. Gardner, H.W., and Jursinic, P.A. (1981) Biochim. Biophys. Acta 665, 100-112.
9. Vioque, E., and Holman, R.T. (1962) Arch. Biochem. Biophys. 99, 522-528.
10. Selke, E., Rohwedder, W.K., and Dutton, H.J. (1975) J. Am. Oil Chem. Soc. 52, 232-235.
11. Gardner, H.W., and Kleiman, R. (1981) Biochim. Biophys. Acta 665, 113-125.
12. Van Os, C.P.A., Vliegenthart, J.F.G., Crawford, C.G., and Gardner, H.W. (1982) Biochim. Biophys. Acta 713, 173-176.
13. Frankel, E.N., Neff, W.E., and Selke, E. (1983) Lipids 18, 353-357.
14. Chan, H.W.-S., Prescott, F.A.A., and Swoboda, P.A.T. (1976) J. Am. Oil Chem. Soc. 53, 572-576.
15. Selke, E., Frankel, E.N., and Neff, W.E. (1978) Lipids 13, 511-513.
16. Gardner, H.W., and Plattner, R.D. (1984) Lipids 19, 294-299.
17. Chan, H.W.-S., Prescott, F.A.A., and Swoboda, P.A.T. (1976) J. Am. Oil Chem. Soc. 53, 572-576.
18. Chan, H.W.-S., Levett, G., and Matthew, J.A. (1979) Chem. Phys. Lipids 24, 245-256.
19. Porter, N.A., Lehman, L.S., Weber, B.A., and Smith, K.J. (1981) J. Am. Chem. Soc. 103, 6447-6455.
20. Dobbs, A.J., Gilbert, B.C., Laue, H.A.H., and Norman, R.O.C. (1976) J. Chem. Soc. Perkin II, 1044-1047.
21. Sessa, D.J., and Plattner, R.D. (1979) J. Agric. Food Chem. 27, 209-210.
22. Lie Ken Jie, M.S.F., and Lam, C.H. (1977) Chem. Phys. Lipids 20, 1-12.

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Fatty Acid Metabolism and Cell Proliferation. V. Evaluation of Pathways for the Generation of Lipid Peroxides

NOBUHIRO MORISAKI, JENIFER A. LINDSEY, JUDITH M. STITTS, HANFANG ZHANG and **DAVID G. CORNWELL**, *Department of Physiological Chemistry, The Ohio State University, Columbus, OH 43210*

ABSTRACT

Primary cultures of smooth muscle cells were established from the medial layer of guinea pig aorta. Confluent cells at passage level 4-6 were challenged with arachidonic acid and treated with a number of antioxidants and inhibitors of specific lipid peroxidation pathways. Lipid peroxidation was measured by the thiobarbituric acid test for malondialdehyde (MDA) and the isolation of hydroperoxy fatty acids (HPETE) by high performance liquid chromatography (HPLC). Prostanoids were measured by radioimmunoassay and the separation of labeled compounds by HPLC. MDA, 6-keto-$PGF_{1\alpha}$, and PGE_2 were formed when cells were challenged with arachidonic acid and these cells synthesized small amounts of one HPETE isomer, 15-HPETE. The HPETE isomers characteristic of the lipoxygenase pathway, 12-HPETE and 5-HPETE, were not detected. Furthermore, the lipoxygenase inhibitors, eicosatetraynoic acid (ETYA) and 6,7-dihydroxycoumarin (Esculetin), did not block MDA formation. These data show that MDA is not generated in the cells by a lipoxygenase pathway. The cyclooxygenase inhibitors, indomethacin and ETYA, did not block MDA formation but these agents blocked the formation of 15-HPETE. These data show both that 15-HPETE is generated by a cooxidation pathway and that 15-HPETE and cooxidation are not involved in MDA formation. Three inhibitors of cytochrome P_{450} linked lipid peroxidation, 2-amino-3-ethoxycarbonyl-6-benzyl-4, 5,6,7-tetrahydrothieno-[2,3-C]-pyridine (Tinoridine), 3-methyl-1,2-di-3-pyridyl-1-propanone (Metyrapone) and phenobarbital, did not block MDA formation. These data support earlier studies that indicated that MDA is not generated by a P_{450} pathway. Cells contained a bound precursor that decomposed to MDA when cells were treated with Fe^{3+}. The cells exhibited autofluorescence and concentric lamellae in lipid droplets that are characteristic of ceroid-lipofuscin. These observations are consistent with lipid peroxidation through increased peroxisomal activity leading to the generation of MDA and the accumulation of ceroid-lipofuscin. The natural antioxidants, vitamin E and vitamin E quinone (EQ), and the synthetic antioxidants, butylated hydroxytoluene and nordihydroguaiaretic acid (NDGA), α-naphthol (α-N) and propyl gallate (PrGa), all blocked MDA formation in confluent smooth muscle cells, showing that these antioxidants did not function solely as specific inhibitors of lipoxygenase, cooxidation or P_{450} pathways. Cell proliferation was measured in cells challenged with arachidonic acid and treated with antioxidants and other inhibitors. The least cytotoxic and most potent antioxidant, EQ, blocked MDA formation in confluent cells and promoted growth in proliferating cells when it was present in either system in the same concentration range. The synthetic antioxidants, NDGA, α-N and PrGa, blocked prostanoid synthesis and promoted growth in proliferating cells. The cyclooxygenase inhibitors, indomethacin, ETYA and Esculetin, did not enhance cell proliferation even though they were highly effective inhibitors of prostanoid synthesis. These data suggest, but do not prove, the hypothesis that cell proliferation is controlled in part by general peroxidation reactions rather than the specific peroxidation reaction involved in prostanoid synthesis.
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INTRODUCTION

Studies from several laboratories show that polyunsaturated fatty acids at concentrations greater than 30 μM inhibit the proliferation of cells in tissue culture (1). Antioxidants (1-7), including the natural antioxidants vitamin E (E) (2-4,7) and vitamin E quinone (EQ) (4-6), and the synthetic antioxidants, butylated hydroxytoluene (BHT) (2,7), α-naphthol (α-N) (2), 6-hydroxy- 2,5,7,8-tetramethylchrom-2-carboxylic acid (2) and dipyridamole (7), block the inhibitory effect of polyunsaturated fatty acids on cell proliferation. Inhibitory polyunsaturated fatty acids generate lipid peroxides that can be estimated by the thiobarbituric acid test (TBA) for malondialdehyde (MDA) (4,5,7,8). Vitamin E, BHT and dipyridamole not only prevent the inhibitory effect of polyunsaturated fatty acids on cell proliferation but also block MDA formation by cells in culture (4,5,7,8). Fatty acid preparations that contain traces of hydroperoxy fatty acids (HPETE) inhibit much more than pure fatty acids and all fatty acid preparations must be monitored for the presence of HPETE

in cell growth studies (1-8). The MDA and HPETE data show that lipid peroxides are involved in the inhibition of cell proliferation.

Lipid peroxides are formed in cells by a number of different mechanisms involving lipoxygenase (1,9-11), cyclooxygenase linked cooxidation (1,12,13) and microsomal cytochrome P_{450} (1,14,15) pathways. Lipoxygenase (16-21), cooxidation (17,18) and P_{450} (22) reactions are found in aorta, smooth muscle cells cultured from aorta and particulate fractions from aorta and cultured cells. Antioxidants, e.g., E and α-N, are effective inhibitors of lipoxygenase (23,24), cooxidation (13) and P_{450} (15) reactions. Data indicating that these antioxidants block MDA formation in smooth muscle cells (4,5,7,8) suggest that lipid peroxidation in these cells may involve either lipoxygenase, cooxidation or P_{450} reactions.

We have examined various lipid peroxidation pathways in smooth muscle cells by measuring the effects of a number of inhibitors on MDA and HPETE formation, prostanoid synthesis and cell proliferation. The synthetic antioxidants, nordihydroguaiaretic acid (NDGA), α-N and propyl gallate (PrGa), were selected because these agents are highly effective inhibitors of soybean lipoxygenase (23,24) and these agents, particularly NDGA (25), have been used as mammalian lipoxygenase inhibitors in many studies. Two agents, eicosatetraynoic acid (ETYA) and 6,7-dihydroxycoumarin (Esculetin: ES), were selected because they inhibit both lipoxygenase and cyclooxygenase. One agent, ETYA, inhibits both enzymes with similar effectiveness (9) whereas the second agent, ES, preferentially inhibits lipoxygenase (26). Indomethacin (IM) was chosen as a specific cyclooxygenase inhibitor (9). 2-Amino-3-ethoxycarbonyl-6-benzyl-4,5,6,7-tetrahydrothieno-[2, 3-C] pyridine (Tinoridine: TO) was selected for study because this agent is 50 times more effective than E as an inhibitor of P_{450} lipid peroxidation reactions (27,28). 3-Methyl-1,2-di-3-pyridyl-1-propanone (Metyrapone: MR) was selected because this agent significantly inhibits P_{450} lipid peroxidation reactions (29). Phenobarbital (PB) was selected for study because this agent competes for reducing equivalents from NADPH-P_{450} reductase during metabolic transformations, inhibiting P_{450} lipid peroxidation (15).

Intracellular lipid peroxidation generates MDA from both free fatty acid derivatives, e.g., HPETE (1,10-19), and bound fatty acid derivatives that are incorporated into complex pigments such as lipofuscin (30-32). We have used lipid extraction and HPLC techniques to find if MDA in smooth muscle cells is formed from intracellular HPETE. These techniques and time studies with Fe^{3+}, a catalyst that promotes MDA formation from lipid peroxides (33,34), have been used to investigate the occurrence of a bound MDA precursor in cells challenged with arachidonic acid [20:4 (n-6)].

MATERIALS AND METHODS

Materials

20:4 (n-6) Was purchased from NuChek Prep (Elysian, MN), purified by elution from a Unisil^R column with hexane/ether (9:1, v/v), and used only when thin layer chromatography (TLC) showed that lipid peroxides were absent (35). Other reagents were obtained from the following sources: vitamin E and vitamin E quinone (Eastman, Rochester, NY); esculetin, indomethacin, α-naphthol, nordihydroguaiaretic acid, phenobarbital, propyl gallate, metyrapone (Sigma, Chemical Co. St. Louis, MO); eicosatetraynoic acid (Hoffman-La Roche, Nutley, NJ); tinoridine (Yoshitomi, Fukuoka, Japan); [1-^{14}C]-20:4 (n-6) (40-60 mCi/mmol) and 12-L-hydroxy-5,8,10,14-eicosatetraenoic acid [5, 6,8,9,11, 12,14,15,-^{3}H(N)] (30-60 Ci/mmol) (New England Nuclear, Boston, MA).

Tissue Culture

Primary cultures of smooth muscle cells were established from the dissected medial layer of guinea pig aorta from prepubertal males (1-8,35,36). The medium for growing cells to confluency (growth medium) was prepared from 1X Eagle's minimum essential medium containing Hank's salts and 24 mM HEPES buffer (GIBCO, Grand Island, NY) supplemented with 50 μg/ml of gentamycin sulfate (Schering, Kenilworth, NJ), 2 mM glutamine, 1X nonessential amino acid (Microbiological Associates, Walkersville, MD), 1 mM sodium pyruvate, and 1.3 mg/ml of sodium bicarbonate. This medium was supplemented with 10% fetal bovine serum (Sterile Systems, Logan UT; Hyclone lot 100348). The medium in lipid peroxidation and prostanoid studies with confluent cells (experimental medium) consisted of growth medium supplemented with 20% fetal bovine serum, 1X essential amino acids, and 1X essential vitamins. Cells from random confluent cultures were detached with trypsin and counted (37). Cells were used at passage levels 4-6. Cloning medium contained 10% fetal bovine serum and the same supplements as experimental medium.

Fatty acids and other agents were dissolved in 95% ethanol and diluted with experimental medium or cloning medium. Control cultures

were treated with medium containing the same amount of 95% ethanol.

TBA Assay

Lipid peroxidation in tissue cultures (4) was measured with cells seeded at 1.3×10^4 cells/cm^2 in flasks containing 4 ml of experimental medium. The cells were grown to confluency before treatments were initialed. Cells were killed and disrupted by the addition of 2 ml of 20% trichloroacetic acid to the tissue culture flask. Four ml of 0.67% TBA was then added to the flask and this mixture was incubated for 20 min at 97 C. The flask contents were decanted and centrifuged at 600 g for 30 min at 4 C. The absorbance of the supernatant was measured at 532 nm and converted to nmol from a standard curve generated with 1,1,3,3-tetramethoxypropane. Lipid peroxides are reported as nmol MDA/culture.

Lipid peroxidation was also studied in a model system that contained 1 mM 20:4 (n-6) in 0.1 M phosphate buffer (pH 7.4). The 20:4 (n-6) was incubated in the presence and absence of 2.84 mM cumene hydroperoxide (CHP) and 41 μM Fe^{3+}. Mixtures were incubated at room temperature for 10 min. The Fe^{3+} was added either at the beginning or at the end of the incubation period. Lipid peroxides were assayed by the TBA procedure (4) and reported as absorbance at 532 nm (A_{532}).

High Performance Liquid Chromatography (HPLC)

HPLC was used to isolate and to identify hydroperoxy fatty acids and prostanoids. The reference compounds 15-hydroperoxyeicosatetraenoic acid (15-HPETE) and 12-hydroxyeicosatetraenoic acid (12-HETE) were synthesized by published procedures using soybean lipoxygenase (38) and human platelets (9), respectively. The 15-HPETE was converted to the hydroxy compound, 15-HETE, by treatment with $SnCl_2$ in methanol (9) and this compound cochromatographed with authentic 15-HETE supplied by Dr. R.W. Bryant. Reference PGE_2 and 6-keto-$PGF_{1\alpha}$ were supplied by Dr. John E. Pike (Upjohn, Kalamazoo, MI). Tritiated 12-HETE was purchased from New England Nuclear (Boston, MA).

Prostanoids and hydroperoxy and hydroxy fatty acids were separated by a published HPLC procedure (39) using a reversed-phase column (Ultrasphere-ODS [5 μm particles], 0.46 i.d. × 25 cm [Beckman, Irvine, CA]) and various mixtures of acetonitrile-aqueous phosphoric acid (pH 2). Column fractions were monitored both by radioactivity and by absorbance at 234 nm. The HPLC instrument used a model 110A pump (Beckman, Irvine, CA), model 110-10 variable-wavelength detector (Hitachi, Tokyo, Japan), model 420 microprocessor (Altex, Berkeley, CA) and model C-RIA integrator.

In tissue culture experiments, fatty acid derivatives were extracted from both media and cells that were first acidified with 5% formic acid to pH 3.5-4.5. Cells and media were homogenized (teflon) after acidification and before extraction. The homogenate was extracted 2 times with 4 vol ethyl acetate. Recoveries were greater than 90% when reference compounds were added to tissue cultures.

Prostaglandin Biosynthesis

6-Keto-$PGF_{1\alpha}$ in tissue culture media was estimated by a standard radioimmunoassay (RIA) procedure (40). The cross-reactivity of the 6-keto-$PGF_{1\alpha}$ antibody was: PGE_2, 0.15%; PGD_2, 0.02%; $PGF_{2\alpha}$, 0.10%; 20:4 (n-6), 0.005%. Data for immunoreactive metabolites are expressed as nmol/culture.

Cell Proliferation

Smooth muscle cells, 3-5 days after confluence, were seeded at low densities (40-80 cells/cm^2) in Falcon single-well (60 × 15 mm) plates. The same number of cells was seeded in control and treatment plates in each experimental series. Cells were allowed to attach to the plastic petri plates for 1 day before initial treatment. Cells were retreated with a media change at day 5 of the incubation period. After an 8-10 day incubation period, cells were fixed in 3.7% phosphate-buffered formalin and stained with filtered Giemsa.

A relative cell count was obtained from the total cell area on the Falcon plate. Total cell area was measured by image analysis using an Optomax Visual Analysis System (Optomax, Wallis, NH). The relationship between cell area and cell number was validated both with a microscope and with a Coulter Counter (6-8). Cells from the same primary culture and the same batch of growth medium were compared in each treatment group.

Statistics

Data are reported as mean ± SEM. The significance of differences in a treatment series was determined by a one-way analysis of variance (F-ratio). Individual treatments were compared with the control by the Tukey-HSD test (sample sizes equal), the Scheffe test (sample sizes unequal) or the Student's *t*-test when only one treatment group was involved.

RESULTS

Lipid Peroxidation in Model Systems

Fatty acid peroxides (TBA reaction) were formed when 20:4 (n-6) was incubated for 10 min in a phosphate buffer at pH 7.4 (Table 1). Ferric ion had no effect on the TBA reaction when MDA was generated from 1,1,3,3-tetramethoxypropane. However, the MDA yield (A_{532}) from lipid peroxides was increased when Fe^{3+} was added either at the beginning or at the end of the incubation period (Table 1). The MDA yield with Fe^{3+} was increased further when the organic hydroperoxide, CHP, was added to the incubation system. The order of Fe^{3+} addition had no effect on the amount of MDA formed in either system. These experiments confirm previous studies that show the catalytic effect of Fe^{3+} on the conversion of lipid peroxides to MDA (33,34).

In a second series of experiments, fatty acid hydroperoxides were extracted from the incubation mixtures, separated by HPLC and detected by their conjugated diene absorbance (A_{234}). Six peaks were formed when 20:4 (n-6) was incubated with either CHP alone, or CHP and Fe^{3+} added at the beginning of the incubation period (Table 2). The Fe^{3+} catalyst had no effect on either the number or size of the peaks (Table 2). These data provide direct evidence that Fe^{3+} does not have a catalytic effect on the formation of lipid peroxides.

Several experiments showed that the synthetic antioxidant, BHT, blocked 20:4 (n-6) peroxidation in the model system. Thus BHT inhibited the formation of the 6 oxidation products separated by HPLC (Table 2) and as a consequence, BHT decreased the MDA yield from 20:4 (n-6) peroxides that was measured by the TBA reaction (Table 3). The MDA yield from 20:4 (n-6) was decreased by E and EQ, showing that these naturally occurring compounds both functioned as antioxidants in the model system (Table 3). The MDA yield from 20:4 (n-6) peroxides was decreased by NDGA and α-N, showing that these synthetic antioxidants also functioned in the model system (Table 3). These naturally occurring and synthetic antioxidants were used in subsequent experiments with cultured smooth muscle cells. One additional synthetic antioxidant, PrGa, was also used in tissue culture experiments, but this agent could not be evaluated in the model system because it formed a colored Fe^{3+} complex that interfered in the TBA test.

Prostanoid and Lipid Peroxide Synthesis in Confluent Cultures of Smooth Muscle Cells

Smooth muscle cells, grown to confluency as described in Methods, contained 1,100,000 ± 139,000 cells/culture. These cells yielded 0.55 ± 0.09 nmol 6-keto-$PGF_{1\alpha}$/culture when they were incubated for 24 hr with fresh media alone and these cells yielded 3.37 ± 0.20 nmol 6-keto-$PGF_{1\alpha}$/culture when they were incubated for 24 hr with fresh media containing 120 μM 20:4 (n-6). Cells and media from cultures incubated with [1-^{14}C]-20:4 (n-6) were separated before lipid extraction and HPLC analysis. The prostanoids 6-keto-$PGF_{1\alpha}$ and PGE_2 were only found in the media.

Confluent cultures yielded 0.96 ± 0.13 nmol MDA/culture when they were incubated for 24 hr with fresh media alone. Media and cells were separated and the media was centrifuged to

TABLE 1

Lipid Peroxidation (TBA Test) in Model Systems

System[a]	A_{532} Mean ± SEM[b]
A. Effect of Fe^{3+}	
20:4 (n-6)	0.059 ±0.003 (33)
20:4 (n-6) + Fe^{3+} (beginning)	0.088 ±0.006 (8)[c]
20:4 (n-6) + Fe^{3+} (end)	0.093 ±0.003 (6)[c]
F ratio	18.0
B. Effect of CHP + Fe^{3+}	
20:4 (n-6) + CHP	0.058 ±0.005 (9)
20:4 (n-6) + CHP + Fe^{3+} (beginning)	0.173 ±0.003 (34)[c]
20:4 (n-6) + CHP + Fe^{3+} (end)	0.182 ±0.007 (6)[c]
F ratio	171.8

[a]The model system (see Methods) contained 1 mM 20:4 (n-6), 2.84 mM CHP and 41 μM Fe^{3+}.

[b]Number of experiments in parentheses.

[c]Differed significantly from control (Scheffe test).

TABLE 2

Separation (HPLC) and Estimation (A_{234}) of Lipid Peroxides in Model Systems

Peak[a] (Elution time)	System[b]	Peak Area[c]
A (28.4 min)	20:4 (n-6) + CHP	1,000,000
	20:4 (n-6) + CHP + Fe^{3+}	1,050,000
	20:4 (n-6) + CHP + Fe^{3+} + BHT	175,000
B (31.7 min)	20:4 (n-6) + CHP	535,000
	20:4 (n-6) + CHP + Fe^{3+}	562,000
	20:4 (n-6) + CHP + Fe^{3+} + BHT	155,000
C (33.0 min)	20:4 (n-6) + CHP	281,000
	20:4 (n-6) + CHP + Fe^{3+}	332,000
	20:4 (n-6) + CHP + Fe^{3+} + BHT	145,000
D (34.5 min)	20:4 (n-6) + CHP	362,000
	20:4 (n-6) + CHP + Fe^{3+}	387,000
	20:4 (n-6) + CHP + Fe^{3+} + BHT	121,000
E (36.1 min)	20:4 (n-6) + CHP	316,000
	20:4 (n-6) + CHP + Fe^{3+}	315,000
	20:4 (n-6) + CHP + Fe^{3+} + BHT	110,000
F (40.1 min)	20:4 (n-6) + CHP	775,000
	20:4 (n-6) + CHP + Fe^{3+}	863,000
	20:4 (n-6) + CHP + Fe^{3+} + BHT	156,000

[a]Peaks were separated by HPLC with 55% acetonitrile-aqueous phosphoric acid (pH 2).
[b]The model systems (see Methods) contained 1 mM 20:4 (n-6), 2.84 mM CHP, 41 μM Fe^{3+} (beginning) and 1,000 μM BHT.
[c]Integrator units.

ensure the removal of all cells. The TBA analysis of these fractions showed that MDA was distributed between media and cells (Table 4). Confluent cultures incubated for 24 hr with fresh media containing 120 μM 20:4 (n-6), yielded 13.8 ± 0.52 nmol MDA/culture. This increment in MDA was found only in cells when media and cells were separated before TBA analysis (Table 4).

Ferric ion increased the MDA yield when it was added to smooth muscle cells incubated in either media alone or media containing 120 μM 20:4 (n-6) (Table 4). The increase in MDA was the same when Fe^{3+} was added either at the beginning or at the end of the incubation period (Table 4), showing that Fe^{3+} in cell cultures catalyzed the decomposition of fatty acid hydroperoxide derivatives to MDA just as Fe^{3+} in model systems catalyzed the decomposition of fatty acid hydroperoxides to MDA (Tables 1-2). Finally, a chloroform-methanol extract of cells in culture yielded MDA when Fe^{3+} was added to the TBA assay system. Thus, studies with the Fe^{3+} catalyst show that MDA is generated from a lipid soluble precursor and the distribution studies show that the MDA precursor is localized in the cells rather than the media.

Isolation of 6-keto-$PGF_{1\alpha}$, PGE_2 and 15-HPETE

Confluent cultures were incubated for 24 hr with labeled [1-^{14}C] 120 μM 20:4 (n-6). Fatty acid derivatives were extracted immediately and separated by HPLC as decribed in Methods. Cells in culture synthesized 6-keto-$PGF_{1\alpha}$ and PGE_2 (Fig. 1). No 12-HPETE, 5-HPETE, 12-HETE or 5-HETE were found in the extract by HPLC. HPLC gave one small peak that co-chromatographed with authentic 15-HPETE (Fig. 1). This peak was radioactive and had an absorbance at 235 nm. The peak was not found when either IM or ETYA was added to the incubation mixture. In addition, mild alkaline hydrolysis followed by extraction and HPLC analysis showed that triglycerides and phospholipids did not contain esterified H(P)ETES. The synthesis of 15-HPETE by cooxidation in particulate fractions of fetal calf aorta and the

TABLE 3

Effect of Antioxidants on Lipid Peroxidation (TBA Test) in the Model System

System[a]	A_{532} Mean ± SEM[b]
Model system alone	0.173 ±0.003 (34)
Model system + 100 μM BHT	0.066 ±0.002 (11)[c]
Model system + 1000 μM BHT	0.040 ±0.002 (6)[c]
F ratio	350.2
Model system + 1 μM E	0.091 ±0.003 (11)[c]
Model system + 10 μM E	0.051 ±0.002 (11)[c]
Model system + 100 μM E	0.051 ±0.003 (11)[c]
Model system + 1000 μM E	0.061 ±0.003 (10)[c]
F ratio	337.2
Model system + 1 μM EQ	0.118 ±0.004 (8)[c]
Model system + 10 μM EQ	0.064 ±0.002 (8)[c]
Model system + 100 μM EQ	0.046 ±0.001 (8)[c]
Model system + 1000 μM EQ	0.046 ±0.001 (8)[c]
F ratio	279.6
Model system + 100 μM NDGA	0.119 ±0.003 (11)[c]
Model system + 1000 μM NDGA	0.069 ±0.002 (8)[c]
F ratio	177.3
Model system + 100 μM α-N	0.147 ±0.005 (11)[c]
Model system + 1000 μM α-N	0.081 ±0.004 (8)[c]
F ratio	100.8

[a]The model system (see Methods) contained 1 mM 20:4 (n-6), 2.84 mM CHP and 41 μM Fe^{3+} (beginning).
[b]Number of experiments in parentheses.
[c]Differed significantly from model system alone (Scheffe test).

TABLE 4

Lipid Peroxidation (TBA Test) in Confluent Cultures of Aorta Smooth Muscle Cells Incubated for 24 Hours

Experiment	MDA nmol/culture[a]
A. Distribution of lipid peroxides	
Media alone	
Media	0.44 ± 0.11 (3)
Cells	0.77 ± 0.05 (3)
Media + 120 μM 20:4 (n-6)	
Media	0.55 ± 0.05 (3)
Cells	12.9 ± 0.27 (3)
B. Effect of Fe^{3+}	
Media alone	1.59 ± 0.13 (4)
Media alone + 10 μM Fe^{3+} (beginning)	3.34 (2)
Media alone + 50 μM Fe^{3+} (beginning)	4.48 ± 0.26 (4)
Media alone + 100 μM Fe^{3+} (beginning)	5.06 (2)
Media alone + 50 μM Fe^{3+} (end)	4.22 (2)
Media + 120 μM 20:4 (n-6)	14.7 ± 0.62 (4)
Media + 120 μM 20:4 (n-6) + 10 μM Fe^{3+} (beginning)	23.7 (2)
Media + 120 μM 20:4 (n-6) + 50 μM Fe^{3+} (beginning)	33.5 ± 0.56 (4)
Media + 120 μM 20:4 (n-6) + 100 μM Fe^{3+} (beginning)	34.4 (2)
Media + 120 μM 20:4 (n-6) + 50 μM Fe^{3+} (end)	33.3 (2)

[a]Mean ± SEM; number of experiments in parentheses.

inhibition of its synthesis by IM was recently reported (17). Our data show that aorta smooth muscle cells also synthesize 15-HPETE and these cells in culture do not convert the HPETE to HETE.

Effect of Antioxidants on 20:4 (n-6) Metabolism

Naturally occurring and synthetic antioxidants block the oxidation of 20:4 (n-6) to its hydroperoxide derivatives and therefore

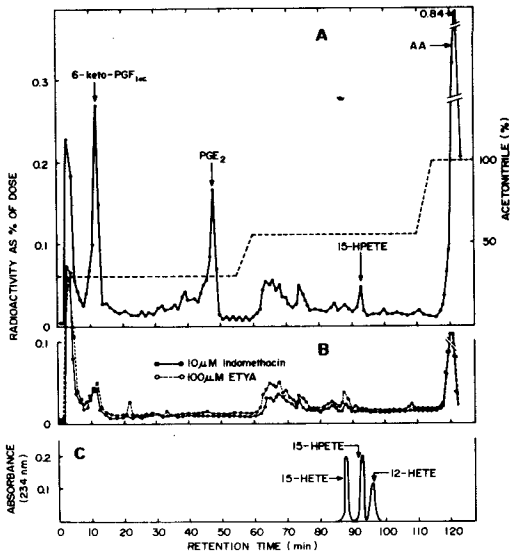

FIG. 1. HPLC separation of prostanoids and lipid peroxides formed in smooth muscle cells and model systems. (A) radioactive 6-keto-$PGF_{1\alpha}$, PGE_2 and 15-HPETE peaks isolated from confluent smooth muscle cells incubated with 120 μM[1-^{14}C]-20:4 (n-6). The 15-HPETE peak also showed a conjugated diene absorbance (234 nm). (B) Absence of prostanoid and lipid peroxide peaks when either 10 μM IM or 100 μM ETYA was added to the incubation system. (C) Separation of reference hydroxy and hydroperoxy compounds (see Methods for syntheses).

diminish the formation of MDA in the TBA assay (Tables 2-3). We showed in previous studies that the natural antioxidants E and EQ and the synthetic antioxidant BHT inhibited MDA formation in smooth muscle cells challenged with 20:4 (n-6) (4,5,7,8). These data were extended in the present study, which showed that E, EQ and BHT selectively blocked MDA formation without altering prostanoid synthesis in the confluent cells (Table 5). Three additional synthetic antioxidants, NDGA, α-N and PrGa, all blocked MDA formation in smooth muscle cells (Table 5). Two of these antioxidants, α-N and PrGa, were effective inhibitors of prostanoid synthesis and one antioxidant, NDGA, inhibited prostanoid synthesis only at high concentrations (Table 5). All naturally occurring and synthetic antioxidants tested in this study inhibited MDA formation in confluent cultures when they were added to the cultures in the 10-100 μM concentration range.

Effect of Cyclooxygenase and Lipoxygenase Inhibitors on 20:4 (n-6) Metabolism

IM was a potent inhibitor of prostanoid synthesis in confluent smooth muscle cells but this cyclooxygenase inhibitor (9) had no effect on MDA synthesis in these cultures (Table 6). ETYA inhibited prostanoid synthesis in confluent smooth muscle cells but a high concentration of this cyclooxygenase and lipoxygenase inhibitor (9) had only a small effect on MDA synthesis in these cultures (Table 6). ES only inhibited prostanoid synthesis at high concentrations and this selective lipoxygenase inhibitor (26) had no effect on MDA synthesis (Table 6). IM and ETYA blocked prostanoid synthesis in confluent smooth muscle cells but a high concentration of this cyclooxygenase and lipoxygenase inhibitor (9) had only a small effect on MDA synthesis in these cultures (Table 6). ES only inhibited prostanoid synthesis at high concentrations and this selective lipoxygenase inhibitor (26) had no effect on MDA synthesis (Table 6). IM and ETYA blocked prostanoid synthesis in confluent cultures when they were added in the 10-100 μM concentration range. Cyclooxygenase and lipoxygenase inhibitors has little effect on MDA formation when they were added in this concentration range.

Effect of Microsomal (Cytochrome P_{450}) Lipid Peroxidation Inhibitors on 20:4 (n-6) Metabolism

One selective P_{450} inhibitor, TO (27,28), only inhibited prostanoid and MDA levels significantly at a high 200 μM concentration (Table 7). A second P_{450} inhibitor, MR (29), had little effect on prostanoid and MDA levels even at a very high 1000 μM concentration (Table 7). Furthermore, PB, an agent that competes with polyunsaturated fatty acids in P_{450} reactions (15), had no effect on either prostanoid or MDA synthesis when it was added over a wide concentration range to smooth muscle cells and the cells were incubated for 4 hr (data not shown) and 24 hr (Table 7).

Effect of Antioxidants and Other Inhibitors on Cell Proliferation

Because the cell density for confluent cultures was 44,000 ± 5,560 cells/cm^2 and cultures were seeded at 40-80 cells/cm^2 in proliferation experiments, the concentrations of antioxidants and other agents were lowered from the 10-100 μM concentration range used with confluent cultures to a 0.1-1.0 μM concentration range for cultures in proliferation experiments. Subsequent proliferation experiments showed that most agents began to exhibit cytotoxicity at 10 μM or greater concentrations.

TABLE 5

Effect of Antioxidant on Prostanoid (6-keto-$PGF_{1\alpha}$) and MDA Formation in Confluent Cultures of Aorta Smooth Muscle Cells Incubated for 24 Hours

Treatment	6-keto-$PGF_{1\alpha}$	MDA
	nmol/culture[a]	
Vitamin E (E)		
120 μM 20:4 (n-6)	2.45 ± 0.28 (6)	12.5 ± 0.41 (6)
120 μM 20:4 (n-6) + 10 μM E	2.70 ± 0.23 (4)	5.2 ± 0.74 (4)[b]
120 μM 20:4 (n-6) + 50 μM E	2.10 ± 0.25 (4)	0.5 ± 0.10 (4)[b]
F ratio	1.035	169.729
Vitamin E quinone (EQ)		
120 μM 20:4 (n-6)	3.90 ± 0.46 (15)	13.9 ± 0.90 (17)
120 μM 20:4 (n-6) + 10 μM EQ	3.48 ± 0.43 (14)	1.8 ± 0.92 (14)[b]
Butylated hydroxytoluene (BHT)		
120 μM 20:4 (n-6)	3.38 ± 0.36 (12)	18.1 ± 1.2 (12)
120 μM 20:4 (n-6) + 10 μM BHT	2.95 ± 0.25 (12)	3.5 ± 0.3 (12)[b]
Nordihydroguaiaretic acid (NDGA)		
120 μM 20:4 (n-6)	2.67 ± 0.27 (8)	13.4 ± 0.60 (6)
120 μM 20:4 (n-6) + 10 μM NDGA	2.81 ± 0.58 (6)	10.9 ± 0.80 (6)[b]
120 μM 20:4 (n-6) + 50 μM NDGA	3.01 (2)	4.7 (2)
120 μM 20:4 (n-6) + 100 μM NDGA	0.81 ± 0.23 (5)[b]	2.3 ± 0.28 (6)[b]
F ratio	5.412	71.444
α-Naphthol (α-N)		
120 μM 20:4 (n-6)	4.03 ± 0.22 (8)	12.8 ± 0.50 (8)
120 μM 20:4 (n-6) + 10 μM α-N	3.30 ± 0.12 (4)[b]	12.4 ± 0.50 (4)
120 μM 20:4 (n-6) + 50 μM α-N	0.11 ± 0.002 (4)[b]	8.3 ± 0.51 (4)[b]
120 μM 20:4 (n-6) + 100 μM α-N	0.03 ± 0.002 (4)[b]	5.5 ± 0.21 (4)[b]
120 μM 20:4 (n-6) + 200 μM α-N	0.03 ± 0.002 (4)[b]	3.0 ± 0.16 (4)[b]
F ratio	136.449	76.672
Propyl Gallate (PrGa)		
120 μM 20:4 (n-6)	4.23 ± 0.25 (6)	11.4 ± 0.10 (6)
120 μM 20:4 (n-6) + 10 μM PrGa	3.92 (2)	12.5 ± 0.20 (4)
120 μM 20:4 (n-6) + 50 μM PrGa	1.49 ± 0.13 (4)[b]	10.0 ± 0.23 (4)[b]
120 μM 20:4 (n-6) + 100 μM PrGa	0.59 ± 0.43 (4)[b]	7.7 ± 0.57 (4)[b]
120 μM 20:4 (n-6) + 200 μM PrGa	0.08 ± 0.009 (4)[b]	5.5 ± 0.23 (4)[b]
F ratio	103.539	99.393

[a]Mean ± SEM; number of experiments in parentheses.
[b]Differed significantly from control (Tukey-HSD test or Student's *t*-test).

We found in previous experiments that the antioxidants, E, EQ and BHT, stimulated cell proliferation when cells were grown in media that was supplemented with a polyunsaturated fatty acid (2-7). These studies were confirmed and extended in the present study. When cells were grown in media that contained 60 μM 20:4 (n-6), proliferation averaged 21% of the proliferation of cells grown in media alone. Low concentrations of NDGA, α-N and PrGa had little effect on cell proliferation when cells were grown in media alone, but these antioxidants had a significant effect, stimulating cells grown in media containing 20:4 (n-6) (Table 8).

Prostanoid levels (RIA assay) were decreased by antioxidants in cell proliferation experiments (Table 8). The TBA test was not sufficiently sensitive for MDA measurements in proliferation experiments, but an effect on MDA levels may be inferred from the prostanoid data. Because the inhibitory effects of NDGA, α-N and PrGa on MDA levels in confluent cultures paralleled or exceeded the inhibitory effects of these antioxidants on prostanoid levels (Table 5) and because these antioxidants inhibited prostanoid synthesis in proliferating cultures, these antioxidant concentrations were probably sufficient to lower MDA levels in proliferating cultures.

The antioxidant effect on proliferating cultures was confirmed in a number of experiments with EQ. This antioxidant is 100 times more effective than the synthetic antioxidants NDGA and α-N in model systems (Table 3) and this antioxidant is even less toxic than E in proliferating cultures (4). Concentrations of EQ as low as the 1-5 μM range (the EQ data did not deviate significantly from a linear trend; F ratio 134.768 for a weighted linear term) inhibited lipid peroxidation in confluent cultures (Table 9) and EQ in the same concentration range stimulated proliferation when cells were grown in media containing 20:4 (n-6) (Table 9).

TABLE 6

Effect of Cyclooxygenase and Lipoxygenase Inhibitors on Prostanoid (6-keto-$PGF_{1\alpha}$) and MDA Formation in Confluent Cultures of Aorta Smooth Muscle Cells Incubated for 24 Hours

Treatment	6-keto-$PGF_{1\alpha}$	MDA
	nmol/culture[a]	
Indomethacin (IM)		
120 μM 20:4 (n-6)	3.00 ± 0.61 (4)	12.6 ± 0.82 (4)
120 μM 20:4 (n-6) + 1 μM IM	1.24 (2)	13.5 (2)
120 μM 20:4 (n-6) + 10 μM IM	0.09 ± 0.01 (4)[b]	13.3 ± 0.99 (4)
120 μM 20:4 (n-6) + 20 μM IM	0.06 (2)	12.2 (2)
120 μM 20:4 (n-6) + 50 μM IM	0.03 (2)	14.6 (2)
Eicosatetraynoic acid (ETYA)		
120 μM 20:4 (n-6)	3.23 ± 0.31 (12)	12.8 ± 0.37 (11)
120 μM 20:4 (n-6) + 10 μM ETYA	1.87 ± 0.29 (8)[b]	11.7 ± 0.52 (8)
120 μM 20:4 (n-6) + 50 μM ETYA	1.12 ± 0.17 (4)[b]	11.3 ± 0.27 (4)
120 μM 20:4 (n-6) + 100 μM ETYA	0.39 ± 0.06 (10)[b]	10.7 ± 0.71 (10)[b]
F ratio	25.617	3.017
Esculetin (ES)		
120 μM 20:4 (n-6)	3.69 ± 0.09 (6)	12.4 ± 0.4 (6)
120 μM 20:4 (n-6) + 10 μM ES	3.84 ± 0.16 (4)	13.1 ± 0.5 (4)
120 μM 20:4 (n-6) + 100 μM ES	2.24 ± 0.04 (6)[b]	13.0 ± 0.5 (6)
120 μM 20:4 (n-6) + 200 μM ES	0.95 ± 0.08 (4)[b]	11.6 (2)
F ratio	43.041	1.077

[a]Mean ± SEM; number of experiments in parentheses.
[b]Differed significantly from control (Tukey-HSD test or Student's *t*-test).

TABLE 7

Effect of Cytochrome P_{450} Peroxidation Inhibitors on Prostanoid (6-keto-$PGF_{1\alpha}$) and MDA Formation in Confluent Cultures of Aorta Smooth Muscle Cells Incubated for 24 Hours

Treatment	6-keto-$PGF_{1\alpha}$	MDA
	nmol/culture[a]	
Tinoridine (TO)		
Media	0.97 ± 0.21 (6)	0.99 ± 0.23 (5)
Media + 10 μM TO	0.81 ± 0.23 (6)	0.73 ± 0.21 (6)
Media + 50 μM TO	0.59 ± 0.11 (4)	0.81 ± 0.36 (4)
Media + 100 μM TO	0.23 ± 0.01 (4)	0.68 ± 0.14 (4)
Media + 200 μM TO	0.04 ± 0.01 (4)[b]	0.82 ± 0.37 (4)
F ratio	4.557	0.178
120 μM 20:4 (n-6)	3.50 ± 0.19 (9)	12.2 ± 0.65 (11)
120 μM 20:4 (n-6) + 10 μM TO	2.95 ± 0.22 (10)	12.8 ± 0.83 (10)
120 μM 20:4 (n-6) + 50 μM TO	2.69 ± 0.15 (7)	12.7 ± 0.46 (8)
120 μM 20:4 (n-6) + 100 μM TO	2.25 ± 0.26 (8)	11.6 ± 0.64 (8)
120 μM 20:4 (n-6) + 200 μM TO	1.30 ± 0.15 (4)[b]	8.6 ± 1.45 (4)[b]
F ratio	9.368	3.150
Metyrapone (MR)		
120 μM 20:4 (n-6)	3.80 ± 0.31 (4)	14.2 ± 0.95 (4)
120 μM 20:4 (n-6) + 10 μM MR	3.28 (2)	12.6 (2)
120 μM 20:4 (n-6) + 100 μM MR	4.21 ± 0.42 (4)	14.6 ± 0.80 (4)
120 μM 20:4 (n-6) + 1000 μM MR	2.87 (2)	15.0 (2)
Phenobarbital (PB)		
120 μM 20:4 (n-6)	4.28 (2)	15.7 (2)
120 μM 20:4 (n-6) + 10 μM PB	4.67 (2)	15.4 (2)
120 μM 20:4 (n-6) + 50 μM PB	4.84 (2)	15.7 (2)
120 μM 20:4 (n-6) + 100 μM PB	5.17 (2)	16.7 (2)
120 μM 20:4 (n-6) + 1000 μM PB	4.94 (2)	16.2 (2)

[a]Mean ± SEM; number of experiments in parentheses.
[b]Differed significantly from control (Tukey-HSD test).

TABLE 8

Effect of Antioxidants on Proliferation and Prostanoid Synthesis with Aorta Smooth Muscle Cells

Treatment	Cell number[a]	6-keto-$PGF_{1\alpha}$[b]
Nordihydroguaiaretic acid (NDGA)		
60 μM 20:4 (n-6)	3,050 ± 214 (8)	28 ± 2.0 (8)
60 μM 20:4 (n-6) + 0.1 μM NDGA	3,690 ± 257 (8)	
60 μM 20:4 (n-6) + 0.5 μM NDGA	6,870 ± 443 (8)[c]	17 ± 0.4 (8)[c]
F ratio	40.76	
α-Naphthol (α-N)		
60 μM 20:4 (n-6)	2,430 ± 288 (7)	21
60 μM 20:4 (n-6) + 1 μM α-N	3.640 ± 391 (7)	11
60 μM 20:4 (n-6) + 5 μM α-N	4,360 ± 454 (8)[c]	6
F ratio	6.21	
60 μM 20:4 (n-6)	5,130 ± 461 (8)	29
60 μM 20:4 (n-6) + 1 μM α-N	7,110 ± 837 (8)	21
60 μM 20:4 (n-6) + 5 μM α-N	8,860 ± 565 (8)[c]	8
F ratio	8.48	
Propyl gallate (PrGa)		
60 μM 20:4 (n-6)	5,010 ± 514 (8)	25 ± 1.2 (8)
60 μM 20:4 (n-6) + 0.05 μM PrGa	8,400 ± 519 (7)[c]	19 ± 1.6 (7)[c]
60 μM 20:4 (n-6) + 0.1 μM PrGa	11,200 ± 1,420 (7)[c]	14 ± 1.1 (8)[c]
F ratio	12.368	18.234

[a]Arbitrary area units; mean ± SEM; number of experiments in parentheses.
[b]Prostanoid levels (pmol/culture) corrected for cell number in pooled (single value) or separate (mean ± SEM) experiments.
[c]Differed significantly from control (Tukey-HSD test or Student's *t*-test).

TABLE 9

Effect of Vitamin E Quinone (EQ) on Lipid Peroxidation (TBA Test) in Confluent Cultures and Cell Proliferation with Aorta Smooth Muscle Cells

Treatment	MDA nmol/culture[a]	Cell number[b]
120 μM 20:4 (n-6)	13.9 ± 0.90 (17)	
120 μM 20:4 (n-6) + 1 μM EQ	11.9 ± 1.32 (8)	
120 μM 20:4 (n-6) + 5 μM EQ	3.7 ± 1.83 (4)[c]	
120 μM 20:4 (n-6) + 10 μM EQ	1.8 ± 0.92 (14)[c]	
120 μM 20:4 (n-6) + 50 μM EQ	0.6 ± 0.06 (6)[c]	
F ratio	35.430	
60 μM 20:4 (n-6)		3,050 ± 214 (8)
60 μM 20:4 (n-6) + 1 μM EQ		4,310 ± 425 (8)[c]
60 μM 20:4 (n-6)		2,430 ± 288 (7)
60 μM 20:4 (n-6) + 1 μM EQ		5,790 ± 569 (8)[c]
60 μM 20:4 (n-6)		5,130 ± 461 (8)
60 μM 20:4 (n-6) + 1 μM EQ		10,900 ± 935 (8)[c]
60 μM 20:4 (n-6)		5,010 ± 514 (8)
60 μM 20:4 (n-6) + 1 μM EQ		8,540 ± 815 (8)[c]
60 μM 20:4 (n-6)		7,200 ± 410 (8)
60 μM 20:4 (n-6) + 1 μM EQ		13,700 ± 672 (7)[c]

[a]Mean ± SEM; number of experiments in parentheses.
[b]Arbitrary area units; mean ± SEM; number of experiments in parentheses.
[c]Differed significantly from control (Scheffe test or Student's *t*-test).

Agents that functioned as cyclooxygenase and lipoxygenase inhibitors did not inhibit lipid peroxidation in confluent cultures (Table 6) and these agents did not stimulate cell proliferation when cells were grown in the presence of 20:4 (n-6) (Table 10). The specific cyclooxygenase inhibitor, IM, showed little cytotoxicity (Table 10). However, the lipoxygenase inhibitors, ETYA and ES, were cytotoxic when their concentrations in proliferating cultures exceeded the 0.1-1.0 μM concentration range (Table 10).

DISCUSSION

Antioxidants in model systems block the formation of lipid peroxides that generate MDA (Table 2-3). The inhibition of MDA formation by natural and synthetic antioxidants (Table 5) that also inhibit soybean and mammalian lipoxygenases (23-25) could be interpreted as evidence for a lipoxygenase pathway leading to hydroperoxy fatty acid synthesis and its breakdown to MDA in guinea pig smooth muscle cells. However, several observations in our study show that the lipoxygenase pathway does not generate MDA in these cells. The metabolites that characterize the lipoxygenase pathway in mammalian cells, 12-H(P)ETE and 5-H(P)ETE (41,42), were not identified previously in cultures of rat smooth muscle cells (43), and these characteristic metabolites were not identified in our studies when intact guinea pig smooth muscle cells were incubated with 20:4 (n-6) (Fig. 1). ETYA and ES are both potent inhibitors of mammalian lipoxygenases (9,26). These agents evidently enter the cell since they block prostanoid synthesis, but these agents have no effect on MDA formation in the cells (Table 6). The formation of only 15-HPETE in trace amounts and the inability of either ETYA or ES to inhibit MDA formation show that significant amounts of MDA are not formed by the lipoxygenase pathway in our smooth muscle cell cultures.

Some investigators have used NDGA as though it functioned solely as an inhibitor of mammalian lipoxygenase (20,42). We find that NDGA, like E and EQ, selectively blocks MDA formation before it blocks prostanoid synthesis (Table 5). The NDGA effect on MDA is obviously unrelated to a lipoxygenase pathway because the studies summarized above show that a lipoxygenase is not involved in MDA formation. Thus, NDGA inhibits several lipid peroxidation pathways and inhibition with NDGA alone does not demonstrate that the lipoxygenase pathway is involved in a specific biological effect.

Microsomal cooxidation reactions during prostanoid synthesis form several HETE isomers (12,13). A particulate fraction from fetal calf aorta synthesizes 15-HETE by this process (17). When guinea pig smooth muscle cells were challanged with [1-^{14}C]-20:4 (n-6), they synthesized a labeled compound with a conjugated diene group (UV absorbance) and this compound had the same elution pattern

TABLE 10

Effect of Cyclooxygenase and Lipoxygenase Inhibitors on the Proliferation of Aorta Smooth Muscle Cells

Treatment	Cell number[a]
Indomethacin (IM)	
90 μM 20:4 (n-6)	10,300 ± 835 (8)
90 μM 20:4 (n-6) + 10 μM IM	9,660 ± 557 (7)
Eiscosatetraynoic acid (ETYA)	
90 μM 20:4 (n-6)	7,700 ± 410 (8)
90 μM 20:4 (n-6) + 0.1 μM ETYA	7,590 ± 528 (8)
90 μM 20:4 (n-6) + 1.0 μM ETYA	6,460 ± 517 (8)
90 μM 20:4 (n-6) + 10 μM ETYA	5,040 ± 152 (8)[b]
90 μM 20:4 (n-6) + 50 μM ETYA	1,490 ± 123 (8)[b]
F ratio	43.592
Esculetin (ES)	
60 μM 20:4 (n-6)	8,790 ± 979 (7)
60 μM 20:4 (n-6) + 0.1 μM ES	6,060 ± 1,280 (5)
60 μM 20:4 (n-6) + 1.0 μM ES	6,520 ± 794 (8)
60 μM 20:4 (n-6) + 10 μM ES	634 ± 226 (7)[b]
F ratio	10.450

[a]Arbitrary area units, mean ± SEM; number of experiments in parentheses.
[b]Differed significantly from control (Tukey-HSD test).

as 15-HPETE on HPLC (Fig. 1). This compound was evidently formed by cooxidation because it was not found when either IM or ETYA was added to the incubation system (Fig. 1). However, cooxidation does not account for MDA formation by smooth muscle cells in tissue culture as neither IM nor ETYA significantly inhibited MDA formation in these cultures (Table 6).

The microsomal cytochrome P_{450} pathway has been found in aorta (22) and this pathway generates lipid peroxides (1,14,15,29). A number of antioxidants, including E and NDGA, inhibit P_{450} oxidation reactions (15,44,45). However, TO is a selective inhibitor of P_{450} lipid peroxidation (27,28) and this agent has little effect on MDA formation even though it partially blocks prostanoid synthesis in our cultures (Table 7). Two other agents, MR (29) and PB (15), are known inhibitors of P_{450} lipid peroxidation and these agents also had no effect on MDA formation by smooth muscle cells (Table 7). Finally, a naphthoquinone, menadione, is an effective inhibitor of P_{450} lipid peroxidation (45,46) and earlier studies from our laboratory showed that menadione did not block MDA formation by smooth muscle cells (4). The TO, MR, PB and menadione data show that MDA is not formed in significant amounts by the P_{450} pathway.

Several observations in our study show that MDA is formed from lipid peroxide derivatives that are contained in complex intracellular lipids rather than the H(P)ETE derivatives of free fatty acids generated by lipoxygenase, cyclooxygenase or P_{450} pathways. The MDA precursors, unlike HETE (42), are not found in media (Table 4) and alkaline hydrolysis experiments show that these precursors are not found as simple esters in triglycerides and phospholipids. The precursors are extracted from cells by chloroform-methanol but the chloroform-methanol extract only generates MDA when Fe^{3+} is added to the TBA reaction mixture. Previous investigators found that Fe^{3+} catalyzed the decomposition of lipid peroxides to MDA in the TBA reaction (33,34). We also find in model systems that Fe^{3+} catalyzes the decomposition of HPETE to MDA in the TBA reaction (Table 1) and the Fe^{3+} does not catalyze the formation of increased amounts of HPETE during lipid peroxidation (Table 2). In tissue culture, the catalytic effect of Fe^{3+} on the TBA reaction is the same when the Fe^{3+} is added either at the beginning or at the end of a 24 hr incubation period (Table 4). The Fe^{3+} effect is dependent on the concentration present (Table 4). Cells in tissue culture evidently contain sufficient Fe^{3+} to catalyze the partial decomposition of bound lipid peroxides to MDA in the TBA reaction.

Previous studies from our laboratory suggest that the bound MDA precursor may be a complex lipid related to ceroid-lipofuscin. We found that smooth muscle cells challenged with 20:4 (n-6) contained increased numbers of acid phosphatase-reactive granules in the perinuclear area (3). Acid phosphatase-reactive granules in the perinuclear region of cultured glial cells have been characterized as lipofuscin (47). Preliminary studies with our cultures show autofluorescence and the presence of concentric lamellae in lipid droplets seen in electron photomicrographs. These lamellae and autofluorescence are characteristic of ceroid-lipofuscin (30-32).

The presence of ceroid-lipofuscin suggests that lipid peroxidation in our cells may be associated with increased peroxisomal activity in these cells. An early study showed that lipofuscin contained a cyanide-insensitive NADPH oxidase (48) and this enzyme is characteristic of peroxisomes (49,50). Early studies showed that lipofuscin was formed in close proximity to peroxisomes (51) and recent studies showed that agents that stimulate peroxisomal activity promote the accumulation of lipofuscin (52). High-fat diets in experimental animals stimulate peroxisomal fatty acid oxidation and this activity decreases the cellular free fatty acid concentration and generates H_2O_2 (53). We suggest that increased peroxisomal activity may occur when cells are challenged with large amounts of polyunsaturated fatty acids, a situation that mirrors in some respects a high-fat diet. Thus, peroxisomal activity could generate bound lipid peroxides that decompose to MDA and ceroid-lipofuscin.

Previous studies from our laboratory showed that lipid peroxides and prostaglandins in the E series both inhibited cell proliferation (1-8). Many fatty acids generate both lipid peroxides and prostaglandins and assessing the contribution of each product to the inhibition of cell proliferation has been difficult. The antioxidants and other inhibitors used in this study bring us closer to a resolution of the problem. One antioxidant, EQ, inhibits MDA formation but has little effect on prostanoid synthesis in confluent cultures (Table 5). This agent is a potent antioxidant (Table 3) that exhibits little cytotoxicity in proliferating cultures (4). Similar concentrations of EQ inhibit MDA formation in confluent cultures and promote growth in cultures challenged with 20:4 (n-6) (Table 9). Three antioxidants, NDGA, α-N and PrGa, inhibit both MDA formation and prostanoid synthesis in con-

fluent cultures (Table 5). These antioxidants also enhance proliferation in cultures challenged with 20:4 (n-6) (Table 8). Cyclooxygenase inhibitors, IM, ETYA and ES, only inhibit prostanoid synthesis in confluent cultures (Table 6) and these agents either have no effect or slightly inhibit cell proliferation (Table 10). These results support, but do not prove, our hypothesis that cell proliferation is controlled in part by general peroxidation reactions rather than the specific peroxidation reactions involved in prostanoid synthesis. Our hypothesis will only be verified by the isolation and identification of inhibitory peroxides in proliferating cultures.

We have proposed in this study that MDA generation in cells challenged with polyunsaturated fatty acids is the result of peroxisomal activity leading to lipid peroxidation and the formation of lipofuscin. A number of studies that are summarized in several reviews (1,30,31, 50,54) note that lipofuscin accumulation is diminished in tumors and other proliferating tissues. This observation has led to the suggestion that lipofuscin somehow diminishes cell proliferation. We suggest that lipofuscin and diminished cell proliferation are both consequences of peroxisomal activity leading to peroxidation reactions.

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REFERENCES

1. Cornwell, D.G., and Morisaki, N. in Free Radicals in Biology, Vol. VI (Pryor, W.A., ed.), pp. 95-148. Academic Press, Inc., NY.
2. Cornwell, D.G., Huttner, J.J., Milo, G.E., Panganamala, R.V., Sharma, H.M., and Geer, J.C. (1979) Lipids 14, 194-207.
3. Miller, J.S., Gavino, V.C., Ackerman, G.A., Sharma, H.M., Milo, G.E., Geer, J.C., and Cornwell, D.G., (1980) Lab. Invest. 42, 495-506.
4. Gavino, V.C., Miller, J.S., Ikharebha, S.O., Milo, G.E., and Cornwell, D.G. (1981) J. Lipid Res. 22, 763-769.
5. Liepkalns, V.A., Icard-Liepkalns, C., and Cornwell, D.G. (1982) Cancer Lett. 15, 173-178.
6. Gavino, V.C., Milo, G.E., and Cornwell, D.G. (1982) Cell Tissue Kinet. 15, 225-231.
7. Morisaki, N., Stitts, J.M., Bartels-Tomei, L., Milo, G.E., Panganamala, R.V., and Cornwell, D.G. (1982) Artery 11, 88-107.
8. Morisaki, N.,Sprecher, H., Milo, G.E., and Cornwell, D.G. (1982) Lipids 17, 893-899.
9. Hamberg, M., and Samuelsson, B. (1974) Proc. Natl. Acad. Sci. USA 71, 3400-3404.
10. Gardner, H.W. (1979) in Autoxidation in Food and Biological Systems, (Simic, M.G., and Karel, M., eds.), pp. 447-504, Plenum Press, NY.
11. Vliegenthart, J.F.G., and Veldink, G.A. (1982) in Free Radicals in Biology, Vol. V (Pryor, W.A., ed.), pp. 29-64, Academic Press, NY.
12. Gale, P.H., and Egan, R.W. in Free Radicals in Biology, Vol. VI, (Pryor, W.A., ed.) Academic Press, NY (in press).
13. Marnett, L.J. (1981) Life Sci. 29, 531-546.
14. Hochstein, P., and Ernster, L. (1963) Biochem. Biophys. Res. Commun. 12, 388-394.
15. Ernster, L., Nordenbrand, K., and Orrenius, S. (1982) in Lipid Peroxides in Biology and Medicine (Yagi, K., ed.), pp. 55-79, Academic Press, NY.
16. Greenwald, J.E., Bianchine, J.R., and Wong, L.K. (1979) Nature 281, 588-589.
17. Powell, W.S. (1982) J. Biol. Chem. 257, 9457-9464.
18. Powell, W.S. (1982) J. Biol. Chem. 257, 9465-9472.
19. Funk, C.D., and Powell, W.S. (1983) Prostaglandins 25, 299-309.
20. Larrue, J., Rigaud, M., Razaka, G., Daret, D., Demond-Henri, J., and Bricaud, H. (1983) Biochem. Biophys. Res. Commun. 112, 242-249.
21. Nakao, J., Ito, H., Chang, W.-C., Koshihara, Y., and Murota, S. (1983) Biochem. Biophys. Res. Commun. 112, 866-871.
22. Juchau, M.R., Bond, J.A., and Benditt, E.P. (1976) Proc. Natl. Acad. Sci. USA 73, 3723-3725.
23. Tappel, A.L., Lundberg, W.O., and Boyer, P.D. (1953) Arch. Biochem. Biophys. 42, 293-304.
24. Panganamala, R.V., Miller, J.S., Gwebu, E.T., Sharma, H.M., and Cornwell, D.G. (1977) Prostaglandins 14, 261-271.
25. Hamberg, M. (1976) Biochim. Biophys. Acta 431, 651-654.
26. Sekiya, K., Okuda, H., and Arichi, S. (1982) Biochim. Biophys. Acta 713, 68-72.
27. Shimada, O., and Yasuda, H. (1977) Biochim. Biophys. Acta 489, 163-172.
28. Yasuda, H., Iaumi, N., Shimada, O., Kobayakawa, T., and Nakanishi, M. (1980) Toxicol. Pharmacol. 52, 407-413.
29. Estabrook, R.W., Chacos, N., Martin-Wixtrom, C., and Capdevila, J. (1982) in Oxygenases and Oxygen Metabolism (Nozaki, M., Yamamoto, S., Ishimura, Y., Coon, M.J., Ernster, L., and Estabrook, R.W., eds), pp. 371-381, Academic Press, NY.
30. Miquel, J., Oro, J., Bensch, K.G., and Johnson, J.E. Jr., (1977) in Free Radicals in Biology, Vol. III, (Pryor, W.A., ed.), pp. 133-182, Academic Press, NY.
31. Strehler, B.L. (1967) in Aspects of the Biology of Ageing (Woolhouse, H.W. ed.), pp. 149-177, Academic Press, NY.
32. Tappel, A.L. (1980) in Free Radicals in Biology, Vol. IV, (Pryor, W.A., ed.), pp. 1-47, Academic Press, NY.
33. Asakawa, T., and Matsushita, S. (1980) Lipids 15, 137-140.
34. Buege, J.A., and Aust, S.D. (1977) Methods Enzymol. 52, 302-310.
35. Huttner, J.J., Gwebu, E.T., Panganamala, R.V.,

Milo, G.E., Cornwell, D.G., Sharma, H.M., and Geer, J.C. (1977) Science 197, 289-291.
36. Huttner, J.J., Cornwell, D.G., and Milo, G.E. (1977) T.C.A. Manual 3, 633-639.
37. Gavino, V.C., Miller, J.S., Dillman, J.M., Milo, G.E., and Cornwell, D.G. (1981) J. Lipid Res. 22, 57-62.
38. Funk, M.O., Isaac, R., and Porter, N.A. (1976) Lipids 11, 113-117.
39. Van Rollins, M., Aveldano, M.I., Sprecher, H.W., and Horrocks, L.A. (1982) Methods Enzymol. 86, 518-530.
40. Levine, L., Gutierrez Cernosek, R.M., and Van Vunakis, H. (1971) J. Biol. Chem. 246, 6782-6785.
41. Nugteren, D.H. (1975) Biochim. Biophys. Acta 380, 299-370.
42. Valone, F.H., Obrist, R., Tarline, N., and Bast, R.C. Jr., (1983) Cancer Res. 43, 197-201.
43. Tansik, R.L., Namm, D.H., and White, H.L. (1978) Prostaglandins 15, 399-408.
44. Rossi, F., Della Bianca, V., and Bellavite, P. (1981) FEBS Lett. 127, 183-187.
45. Slater, T.F. (1979) in Oxygen Free Radicals and Tissue Damage, Ciba Found. Symp. 65 (Fitzsimons, D.W., ed.), pp. 143-159, Excerpta Med., Amsterdam, Netherlands.
56. Talcott, R.E., Shu, H., and Wei, E.T. (1979) Biochem. Pharmacol. 28, 665-671.
47. Collins, V.P., and Brunk, U.T. (1976) Mech. Ageing Develop. 5, 193-207.
48. Bjorkerud, S. (1963) J. Ultrastruct. Res. Suppl. 5, 1-49.
49. Böck, P., Kramar, R., and Pavelka, M. (1980) Peroxisomes and Related Particles in Animal Tissues, Springer-Verlag, Vienna, Austria.
50. Chance, B., Sies, H., and Boveris, A. (1979) Physiol. Rev. 59, 527-605.
51. Novikoff, A.B., Novikoff, P.M., Quintana, N., and Davis, C. (1973) J. Histochem. Cytochem. 21, 1010-1020.
52. Reddy, J.K., Lalwani, N.D., Reddy, M.K., and Qureshi, S.A. (1982) Cancer Res. 42, 259-266.
53. Osmundsen, H. (1982) Ann. N.Y. Acad. Sci. 386, 13-27.
54. Apffel, C.A. (1978) Prog. Exp. Tumor Res. 22, 317-362.

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Thermodynamic Characterization of the Pretransition of Unilamellar Dipalmitoyl-Phosphatidylcholine Vesicles

DOV LICHTENBERG,[b] MOSHE MENASHE,[a] STEVE DONALDSON[a] and RODNEY L. BILTONEN,[a] *[a]Departments of Biochemistry, Pharmacology, and Biomedical Engineering, University of Virginia School of Medicine, Charlottesville, Virginia 22908; and [b]Department of Physiology and Pharmacology, Tel Aviv University, Sackler School of Medicine, Ramat Aviv, Tel Aviv 69978 Israel*

ABSTRACT

High-sensitivity differential scanning calorimetry of small unilamellar vesicles (SUV) made by sonication of dipalmitoylphosphatidylcholine (DPPC) reveals that the gel-liquid crystalline transition at 37 C is preceded by a pretransition at 28 C that is relatively slow ($t_{1/2} \cong$ 2-4 min) and has an enthalpy change of ca. 0.2 kcal/mol. On incubation at 4 C, these SUV fuse spontaneously into large unilamellar vesicles (LUV). LUV also exhibit both a pretransition and a main-phase transition. The temperature of the main transition (T_m) and the enthalpy change of both the pretransition and main transition of these fused vesicles are similar to those of large multilamellar vesicles (MLV). The enthalpy change associated with the transition at 28 C decreases in SUV in a manner directly correlated to the decrease in the apparent enthalpy change of the 37 C main transition, indicating that the smaller (low temperature) transition is indeed a pretransition that is an inherent property of SUV. Therefore, unilamellar vesicles of DPPC appear to exhibit a pretransition at a temperature that varies from 28 C for the small vesicles to 35 C for the much larger vesicles.
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INTRODUCTION

In addition to the main gel-to-liquid crystalline phase transition, a second thermotropic transition, the pretransition, occurs at a lower temperature in multilamellar vesicles (MLV) of synthetic phospholipids (PL) (1,2). X-ray diffraction studies of MLV have shown that in the temperature range between the pretransition temperature (T_p) and the main transition temperature (T_m), the PL bilayer assumes a phase ($P_{\beta'}$) that is distinctly different from the gel phase and the liquid-crystalline phase (3). The question of whether or not a pretransition (and thus a $P_{\beta'}$ phase) also exists in single-walled vesicles made of synthetic lipids is not yet resolved. Electron diffraction studies of multilamellar and unilamellar vesicles (4) concluded that a $P_{\beta'}$ phase does not exist in unilamellar vesicles. This conclusion is consistent with the apparent absence of a pretransition in the calorimetric scans of dipalmitoylphosphatidylcholine (DPPC) large unilamellar vesicles (LUV) made by ethanol injection (5) or by spontaneous fusion of small unilamellar vesicles (SUV) (6). In contrast to these studies, Dufour et al. (7) have presented calorimetric data that indicate 2 transitions in dimyristoylphosphatidylcholine (DMPC) unilamellar vesicles and Friere et al. (8) have presented similar calorimetric data for LUV made of DPPC according to the procedure used by Wong et al. (6). Although in both these reports, the authors referred to the low temperature as a "pretransition," it has not been completely characterized.

Recently, a systematic study of LUV made of DPPC using the reverse-phase evaporation method (9) revealed a "broadened endotherm below the main transition" (10). In addition, this study presented electron microscopic evidence for the existence of a $P_{\beta'}$ phase in LUV in the temperature range between this broadened endotherm and the main transition (10). This observation agrees with earlier studies (11), which have indicated the possible existence of a $P_{\beta'}$ phase in LUV made by the ether injection method (12). Nevertheless, Düzgünes et al. (10) did not reach a definite general conclusion regarding the existence of a pretransition in unilamellar vesicles because they could not exclude the possibility that the existence of a $P_{\beta'}$ phase, indicated by the electron microscopic data and the broadened endotherm below T_m, was caused by oligolamellar vesicles present in their LUV preparations (9). They have, however, noted the absence of such a transition in several previously studied vesicle preparations (5,6) and attributed it to the small size of these vesicles and to the very low enthalpy associated with the pretransition. Thus, the possibility that a pretransition exists in SUV, but the techniques used to investigate these vesicles are not sufficiently sensitive to disclose it, cannot be excluded. However, one may argue that a pretransition only occurs in

MLV and LUV but not in the much smaller and highly curved SUV.

In 1976, the results of Suurkuusk et al. (13) indicated the possible existence of several thermotropic transitions in DPPC preparations consisting of what we now know to be a mixture of small unilamellar vesicles and large unilamellar vesicles (13-15). However, because of the lack of detailed structural knowledge of such systems at that time and problems with the calorimetric baseline, no detailed interpretations of the data on the smaller transitions was made. We have now expanded calorimetric studies of this system by using higher lipid concentrations (~50 mM), a new scanning calorimeter with improved sensitivity and the capability of operating the calorimeter in the cooling mode. The results reported here strongly support the existence of a pretransition in DPPC unilamellar vesicles, both large and small.

EXPERIMENTAL PROCEDURES

L-α-dipalmitoylphosphatidylcholine (DPPC) was purchased from Avanti and used without further purification. Its purity was confirmed by TLC on silica gel using $CHCl_3/CH_3OH/H_2O$ (65:35:4 v/v) as the eluting solvent.

Dispersions of large multilamellar liposomes were prepared by the method of Bangham et al. (16). SUV of DPPC were prepared as described by Suurkuusk et al. (13), except that the sonication was done with a bath sonicator for 1 hr at 45 C. Dispersions were fractionated from larger liposomes by high-speed centrifugation at $10^6 \times g$ for 1 hr. The resultant vesicles (17) were kept above T_m, to avoid fusion (14). LUV of an average diameter of 950 Å were prepared by incubation of SUV at 4 C (6). The lipid concentration of each dispersion was measured as inorganic phosphate according to Barlett's method (18). At least 5 phosphate determinations were made on each sample with a resulting standard deviation of 2%. DPPC concentrations in dispersions examined calorimetrically were 25-50 mM.

The custom-built differential scanning calorimeter used in these experiments was originally designed only for heating scans. Recently, a PDP 11/10 computer was added to the calorimeter to make the control of the scanning rate more precise. The control of the rate of heating is accomplished as follows: the computer monitors the heat-sink temperature and calculates the current scanning rate, using least squares analysis. Next, the computer determines the current scanning error, SCNERR, and the integral of the past errors, ERRSUM. The new voltage setting for the heat-sink power supply is then found using the formula: Volts = $K_1 \times$ SCHERR + K_2 ERRSUM, where K_1 and K_2 are empirically derived feedback coefficients. The addition of computer control makes it possible to use this calorimeter for precisely controlled cooling scans. In the cooling mode, the heater to the adiabatic shield is turned off and cooling initiated. The rate of cooling is then controlled by adjusting the heater to oppose the cooling to maintain a constant rate of cooling. The maximal cooling rate (−9 C/hr) of this instrument is limited by the capacity of the cooling coils.

The apparent excess heat-capacity curves reported in this study have not been corrected for the time response of the instrument. This correction only provides for a true shape of the heat-capacity curve and does not affect the measured enthalpy change or the main phase-transition temperature (19).

The calorimeter was calibrated electrically, as described previously (13). The baseline noise of this instrument is ca. 25 μcal/deg at the scanning rates used in these experiments. At the concentrations used in these studies, the noise amounts to ca. 10^{-3} kcal/mol. This noise level is within the pen width of the attached drawings (except in Fig. 3).

RESULTS AND DISCUSSION

A major problem in obtaining thermodynamic information on the phase behavior of SUV is that most temperature-scanning calorimeters of the required sensitivity operate only in the heating mode. Because the SUV fuse at temperatures below the T_m (13-15), the heat-capacity curve for a system consisting only of SUV is difficult to obtain without the presence of contaminating vesicles of larger sizes (13). Thus, SUV made of DPPC and characterized in the heating mode (Figure 1a), show multiple endothermic phase transitions with maxima at ca. 29 C, 38 C and 42 C. The 2 transitions observed at 38 C and 42 C are the main gel → liquid-crystalline DPPC phase transitions for SUV and for large fused vesicles (13).

On incubation of the SUV at room temperature, spontaneous fusion occurs (14,15). Consequently, the apparent heat capacity associated with the main transition of the fused vesicles increases with time at the expense of the heat capacity of the SUV. This is demonstrated in Figure 1b, which shows the calorimetric scan obtained using a SUV sample that had been incubated at room temperature for 72 hr. The transition observed in this scan at 38-39 C is presumably caused by the melting of a residual amount of SUV. The small but

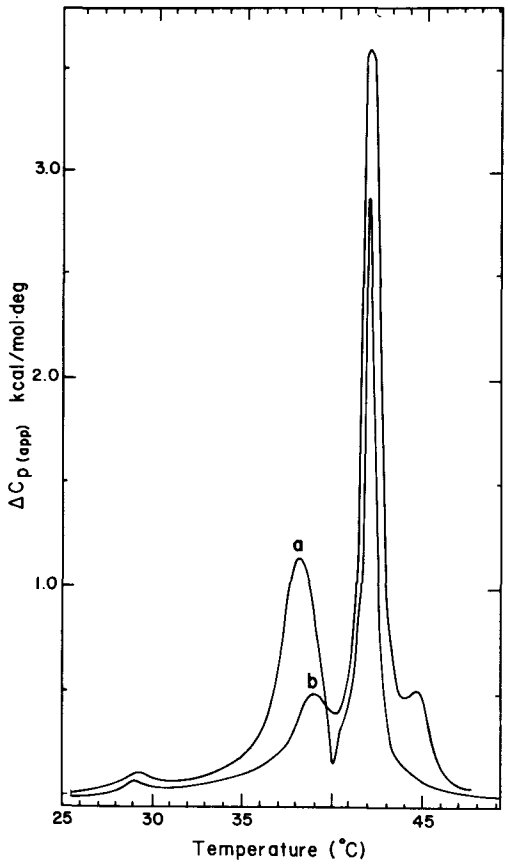

FIG. 1. Heating calorimetric scans of dipalmitoylphosphatidylcholine (DPPC) sonicated small unilamellar vesicles (SUV). DPPC SUV at a concentration of 38.4 ± 0.4 mM in 20 mM TES buffer pH 8.0 were incubated at room temperature for (a) 1.5 hr; (b) 72 hr. Curve a has been vertically displaced slightly in order to demonstrate the details of each curve. Before and during the preparation procedure, the vesicles were maintained above the T_m of DPPC multilamellar vesicles.

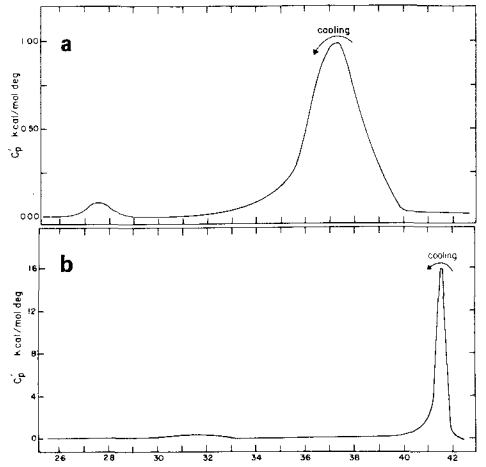

FIG. 2. (a) Cooling calorimetric scan of DPPC SUV. The lipids, at a concentration of 30 ± 0.5 mM in 20 mM TES buffer (pH 8.0), were kept above the T_m during the entire preparation procedures. The scan was performed in the cooling mode starting from 48 C with a scanning rate of −7 C/hr. (b) Cooling calorimetric scan of DPPC LUV. These vesicles were formed spontaneously by fusing small unilamellar vesicles by being kept at 4 C for 1 mo. The lipid concentration is 25 ± 1.1 mM in 20 mM TES buffer, pH 8.0. The vesicles were scanned in the cooling mode at a scanning rate of −7 C/hr.

discernible transition of 29 C obtained in Figures 1a and 1b have not been studied previously. The higher temperature transition at ca. 45 C has not previously been observed. In a subsequent heating scan, it did not appear and it is probably associated with aggregation-disaggregation processes of the lipid vesicles.

SUV are stable for many hours above T_m (13-15). Therfore, we adapted our calorimeter to provide accurate data when operating in the cooling mode. A typical heat-capacity curve obtained in the cooling mode for a freshly prepared sample of SUV is shown in Figure 2a. Two discernible phase transitions are observed. The higher temperature transition is centered at 37.2 C, that is ca. 4 C lower than in MLV, whereas the lower temperature transition is observed at 27.5 C, which is ca. 7 C lower than the pretransition detected for MLV (Table 1). The transition enthalpies of the lower and the higher temperature transitions are estimated to be 0.13 and 5.5 kcal/mol. The latter value is in reasonably good agreement with the value of 6.2 kcal/mol for the main transition of SUV determined by Suurkuusk et al. (13) and the result of Gaber and Sheridan (20), who obtained a value of 6.1 kcal/mol for SUV (see footnote to Table 1).

Incubation of SUV at 4 C for 1 month results in the formation of fused, large unilamellar vesicles with a diameter of ca. 950 Å, with very few SUV remaining (6). When such a preparation is examined by high-sensitivity differential scanning calorimetry in the cooling mode, the endothermic transition profile of the fused vesicles displays 2 transitions centered at 41 C and ca. 32 C, as shown in Figure 2b. The smaller transition at the lower temperature is probably the pretransition of LUV. Our result is consistent with the observations of Dufour et al. (7) and Friere et al. (8), who have shown the existence of a low-temperature transition in LUV composed of DMPC and DPPC.

TABLE 1

Thermodynamic Characteristics of Various Liposome Dispersions

Preparation	Pretransition		Main transition	
	T_m (°C)	ΔH_1 (kcal mol^{-1})	T_m (°C)	ΔH_2 (kcal mol^{-1})
SUV	28.0	0.13 ± 0.03	37.2 ± 0.1	5.5 ± 0.5
LUV	33.8	0.60 ± 0.12	41.4 ± 0.1	7.5 ± 0.5
MLV	35.3	1.80 ± 0.30	41.3 ± 0.1	8.0 ± 0.5

ΔH_2 vary by up to ±10% from one batch of lipid to another. We do not understand the reason for this variation. However, ΔH_1 and ΔH_2 values are very consistent for a given preparation, and the differences in the enthalpy changes as reported in this table among SUV, LUV and MLV exist for any lipid source. The values of ΔH_1 and ΔH_2 reported in this table are for a single preparation of lipid and thus the differences in enthalpy change between SUV, LUV and MLV are reliable within the precision stated.

In cooling scans, distinct thermal transitions are observed at 27-29 C for SUV (Fig. 2a) and at 31-33 C for LUV (Fig. 2b). If, as we suspect, the 27-29 C peak observed in Figure 2a and Figure 1 is a pretransition of the SUV, whose main transition is at 37-39 C, it should exhibit a direct correlation with the latter peak. In order to test this hypothesis, a series of apparent excess heat-capacity curves were obtained with aliquots from the same sample, which had been incubated at room temperature (≃22 C) for varying lengths of time.

The apparent excess heat-capacity data obtained from these experiments, emphasizing the 27-29 C transition and 37-39 C transition, are shown in Figures 3a-3b, respectively, on scales greatly expanded compared with Figure 1. The results on the 27-29 C transition are particularly difficult to analyze because this small transition is superimposed on the leading edge of a much larger transition at 37 C. The enthalpy change associated with the 27-29 C transition was estimated by using a baseline, as shown in Figure 3a. This procedure is obviously not exact but is sufficiently precise for our purposes. The remainder of the data, including those relating to the 41 C transition, were decomposed to yield the apparent enthalpy change associated with each transition in the manner described by Suurkuusk et al. (13). These authors have shown that a linear relationship with negative slope exists between the enthalpy change of the 37 C and 41 C transitions for this system. Our results, plotted in Figure 4, show a linear relationship, with positive slope, between the enthalpy changes of the 27-29 C and the 37-38 C transitions and a linear relationship, with negative slope, between the enthalpy changes associated with the 27-29 C and the 41 C transitions. Given that the 37-38 C transition is associated with the gel-to-liquid transition of small unilamellar vesicles,

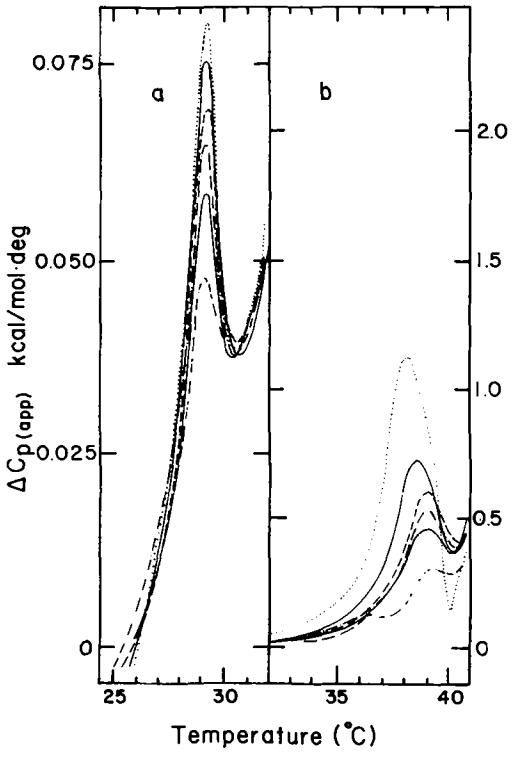

FIG. 3. Heating calorimetric scans of DPPC SUV as a function of their incubation time at room temperature. Heating scans of separate aliquots from one SUV preparation held at room temperature for 1 hr, 14 hr, 30 hr, 46 hr, 72 hr and 23 days are presented in the figure from top to bottom. The scans between 25-32 C: (a) (including the 27-29 C transition) are magnified 30 times compared with (b) the scan in the temperature range of 32-42 C. The dashed (---) baseline for the 27-29 C transition was drawn so that it connects the local minimum in the heat-capacity curve at about 30 C with the point at which the curve departs from its baseline (at ca. 25 C).

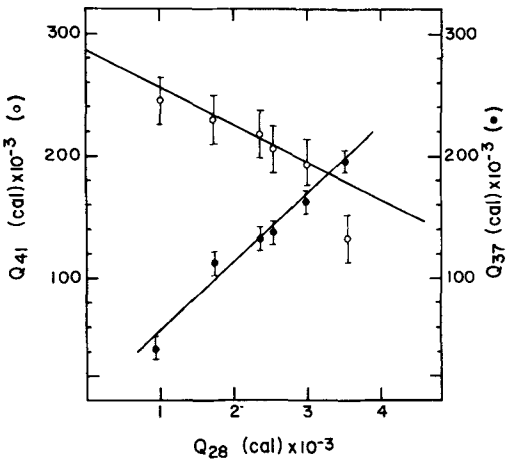

FIG. 4. Correlation between the enthalpy changes corresponding to the transition at 28 C, Q_{28}, to the transitions at 37 C, Q_{37}, (•) and at 41 C, Q_{41}, (○). The enthalpy changes estimated for the 28 C transition shown in Figure 3a are plotted against data obtained from analogous scans shown for the 37 C transition (Fig. 3b). and for the 41 C transition (data not shown). The enthalpy changes were estimated as described in the text, and are reported in actual calories rather than kcal/mol lipid.

the 27-29 C transition is a phenomenon associated with them as well.

We cannot develop a similar correlation between the 32 C transition and the 41 C transition of LUV (Fig. 2b) because the former is always dominated by the gel-liquid crystalline transition of the SUV in mixtures. However, because of the existence of a pretransition in SUV and MLV, we suggest that the 32-34 C transition, found in essentially pure preparations of LUV, is also a pretransition.

The kinetics associated with the pretransition in multilamellar vesicles have been deduced by repeated heating and cooling of the dispersions (19,21). These studies clearly demonstrate that the rate of lipid phase transformation at the pretransition is slower than the transformation that occurs at the main-phase transition temperature. If a similar situation exists with the small unilamellar vesicles, then the apparent T_m of the 27-29 C transition should depend on the scanning rate of the calorimeter. Indeed, a scanning rate dependence of T_m on both heating and cooling rate for the SUV was observed. From the dependence of the apparent T_m on scanning rate we estimate, in a manner similar to Lentz et al. (19), that the true T_m for the low temperature transition is ca. 28 C. The apparent half-time of the transition is ca. 2-4 min, which is slightly faster than the rate of the pretransition in large multilamellar liposomes.

In the course of these studies, we also noted a transition at 16-21 C. However, the appearance and temperature of this transition depended on the history of the sample. This transition was not observed when a vesicle preparation was studied in the heating mode after being incubated at 4 C for 1 mo. However, when the vesicles were heated above the T_m and then cooled to 4 C and stored there for 24 hr, this transition appeared. If vesicles were first heated, then cooled to 4 C and immediately heated, they did not exhibit this transition. This phenomenon appears to be similar to the transition observed initially by Chen and coworkers (22) with multilamellar DPPC liposomes. PL bilayers appear to undergo a number of reversible transitions and, in addition, after proper manipulation of the experimental history of the sample, some irreversible transitions.

In summary, multilamellar vesicles, small unilamellar vesicles and large unilamellar vesicles all exhibit, in addition to the main gel-to-liquid crystalline transition, a pretransition, which occurs ca. 7-9 C below T_m. The thermodynamic characteristics of these transitions for each type of preparation are summarized in Table 1. While the structural change of lipid molecules in the multilamellar liposomes accompanying the pretransition has been characterized, the low temperature transition in SUV and LUV has not. Whether or not all the pretransitions reflect the same type of structural change is still open to question and beyond the scope of this study. Nonetheless, the results show a direct relationship between the melting temperatures and enthalpy changes of the pretransition and main transition and the average vesicle size. The radius of curvature has a definite effect on the thermodynamic changes associated with all the phase transitions of DPPC bilayers.

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REFERENCES

1. Mabrey, S., and Sturtevant, J.M. (1978) Methods Membr. Bio. 9, 237-274.
2. Biltonen, R.L., and Freire, E. (1978) CRC Biochem. 5, 85-124.
3. Janiak, M.J., Small, D.M., and Shipley, G.G. (1976) Biochemistry 15, 4575-4580.
4. Hui, S.W. (1976) Chem. Phys. Lipids 16, 9-18.

5. Takemoto, H., Inoue, S., Yasunaga, T., Sukigara, M., and Toyoshima, Y. (1981) J. Phys. Chem. 85, 1032-1037.
6. Wong, M., Anthony, F.H., Tillack, T.W., and Thompson, T.E. (1982) Biochemistry 21, 4126-4132.
7. Dufour, J., Nunnally, R., Buhle, L., and Tsong, T.Y. (1981) Biochemistry 19, 5576-5594.
8. Friere, E., Markello, T., Rigell, C., and Holloway, P.W. (1983) Biochemistry 22, 1675-1680.
9. Szoka, F., and Papahadjopoulos, D. (1978) Proc. Natl. Acad. Sci. USA 75.
10. Düzgünes, N., Wilschut, J., Keelung, H., Fraley, R., Perry, C., Friend, D.S., James, T.L., and Papahadjopoulos, D. (1983) Biochem. Biophys. Acta 732, 289-299.
11. Copeland, B.R., and McConnell, H.M. (1980) Biochim. Biophys. Acta 599, 95-109.
12. Deamer, D., and Bangham, A.D. (1976) Biochim. Biophys. Acta 443, 629-634.
13. Suurkuusk, J., Lentz, B.R., Barenholz, Y., Biltonen, R.L., and Thompson, T.E. (1976) Biochemistry 15, 1393-1401.
14. Schullery, S.E., Schmidt, C.F., Felgner, P., Tillack, T.W., and Thompson, T.E. (1980) Biochemistry 19, 3919-3923.
15. Schmidt, D.F., Lichtenberg, D., and Thompson, T.E. (1981) Biochemistry 20, 4792-4797.
16. Bangham, A.D., Standish, M.M., and Watkins, J.C. (1965) J. Mol. Biol. 13, 238-252.
17. Barenholz, Y., Gibbs, D., Litman, B.J., Goll, J., Thompson, T.E., and Carlson, F.D. (1977) Biochemistry 16, 2806-2810.
18. Barlett, G.R. (1959) J. Biol. Chem. 234, 466-470.
19. Lentz, B.R., Freire, E., and Biltonen, R.L. (1978) Biochemistry 17, 4475-4480.
20. Gaber, B.P., and Sheridan, J.P. (1982) Biochim. et Biophys. Acta 685, 87-93.
21. Akiyama, M., Terayama, Y., and Matsushima, M. (1982) Biochim. Biophys. Acta 687, 637-639.
22. Chen, C.S., Sturtevant, J.M., and Gaffney, B.J. (1980) Proc. Natl. Acad. Science USA 77, 5060-5063.

Differences in Polyisoprenoid Alcohols of Mono- and Dicotyledonous Seeds

KOTHAPALLI RAVI, JACK W. RIP and **KENNETH K. CARROLL**[1], *Department of Biochemistry, University of Western Ontario, London, Ontario, Canada, N6A 5C1*

ABSTRACT

A lipid fraction enriched in polyisoprenoid alcohols was prepared from seeds of a number of crop plants, using Florisil chromatography. Analysis by HPLC of the fraction from soybeans showed a series of peaks corresponding to α-saturated homologues (dolichols) from 15 to 22 isoprene units in length. Similar results were obtained with seeds of other dicotyledonous species (rapeseed, peanuts, mung beans, navy beans and peas). In contrast, analysis of the seeds of monocotyledonous plants (wheat, rye, barley, rice and corn) by HPLC gave split peaks, indicating the presence of nearly equal amounts of 2 different homologous series of compounds. The polyisoprenoid material isolated from wheat germ was subsequently shown to consist of a mixture of dolichols and α-unsaturated homologues (polyprenols). Treatment with manganese dioxide selectively oxidized the polyprenols to the corresponding aldehydes, which were separated from the dolichols by TLC. The identity of the components was established by infrared–nuclear magnetic resonance (IR-NMR) spectroscopy and by comparison with authentic standards on high performance liquid chromatography (HPLC). The concentration of polyisoprenoid alcohols in seeds varied from 1-16 mg/100 g. Seeds of different species showed some differences in the pattern of homologues present.
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INTRODUCTION

Polyisoprenoid alcohols appear to be universally present in bacteria, plants and animals. They are of interest because they participate in the biosynthesis of cell-wall polymers in bacteria and asparagine-linked glycoproteins in yeasts, plants and animals (1,2).

In some of these compounds, every isoprene unit is unsaturated, (Fig. 1a), whereas in others the α-isoprene unit containing the primary alcohol is saturated (Fig. 1b). The latter type of compound was first isolated from pig liver (3) and was named dolichol from the Greek *dolikos* (long). Subsequent investigation showed that dolichol normally occurs in nature as a series of homologues of varying chain length, so speaking of a mixture of dolichols is perhaps more appropriate.

The term polyprenol has been used collectively for all polyisoprenoid alcohols, regardless of whether the α-isoprene unit is saturated or unsaturated (1,4). No convenient term is available to refer specifically to α-unsaturated polyisoprenoid alcohols. We therefore propose that the short form, polyprenol, be restricted to this class of compound to distinguish it from the α-saturated dolichols (Fig. 1). Where no distinction is made between polyprenols and dolichols, they can be referred to as polyisoprenoid alcohols. Dolichols may also be classified as dihydropolyprenols because the name hexahydropolyprenols was suggested by Hemming (1) for the more highly saturated derivatives isolated from *Aspergillus*.

Dolichols have been isolated mainly from animal tissues, whereas polyprenols are more characteristic of bacteria and plants (1,5). However, a few reports have been made on the occurrence of polyprenols in animals (6,7) and of dolichols in plants (1,8). This paper presents data on the dolichols and polyprenols in seeds of a number of common crop species. All seeds examined contained dolichols, but polyprenols were found only in seeds of cereal grains, which contained dolichols and polyprenols in approximately equal amounts.

MATERIALS AND METHODS

[1-^{3}H] Dolichol (specific radioactivity 12.5 Ci/mmol) was obtained from New England Nuclear, Boston, MA. Human liver dolichol and the individual homologue containing 18 isoprene units (dolichol-18) were isolated and purified by high performance liquid chromatography (HPLC) as described previously (8). Polyprenol-18 from *Prunus avium* was obtained from Calbiochem, San Diego, CA. Silica Gel 60H and Florisil were from E. Merck, Darmstadt, Germany and Fisher Scientific, Toronto, Ontario, Canada, respectively. Chloroform-d was from Merck, Sharp and Dohme, Montreal, Quebec, Canada. Seeds were purchased from local seed stores. All other chemicals were from Fisher Scientific, Toronto, Ontario, Canada.

[1]Career Investigator of the Medical Research Council of Canada.

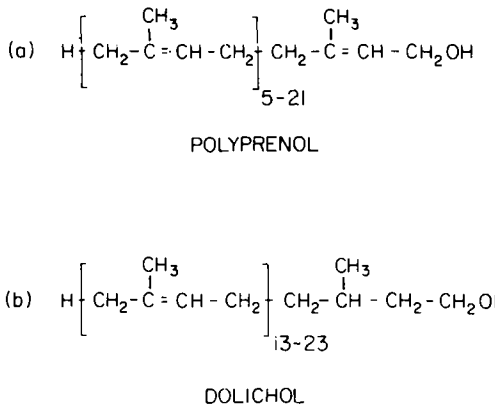

FIG. 1. Structures of polyisoprenoid alcohols.

Isolation of Polyisoprenoid Alcohols from Seeds

Finely ground seeds (10 g) were digested by refluxing for 10 hr in a mixture of 150 ml 30% w/v KOH, 30 ml ethanol and 0.5 g pyrogallol (8). Nonsaponifiable lipids were extracted with diethyl ether and washed several times with water. They were concentrated in a rotary evaporator and chromatographed on columns of Florisil deactivated by 7% (w/v) water (9). Columns were washed with hexane and sequentially eluted with hexane/5% diethyl ether and hexane/15% diethyl ether. Polyisoprenoid alcohols appear in the 15% ether fraction.

HPLC of Polyisoprenoid Alcohols

Fractions enriched in polyisoprenoid alcohols were injected into a Hewlett-Packard 1084-B HPLC equipped with either an analytical (Hewlett-Packard) or semipreparative (Whatman) C18 reverse-phase column. Operating conditions have been described previously (8). Polyisoprenoid alcohol concentrations indicated (Table 1) represent the sum of the homologues containing 13-22 isoprene units, and were corrected for losses incurred during extraction and isolation by using [1-^{3}H] dolichol as tracer.

Oxidation of Polyprenols

Manganese dioxide was prepared as described by Attenburrow et al. (10). Polyisoprenoid alcohols (100 mg) purified from wheat germ by HPLC were dissolved in 10 ml chloroform and treated with 1 g MnO_2 at 4 C for 16 hr.

Nuclear Magnetic Resonance (NMR) and Infrared (IR) Spectra

NMR spectra were recorded in $CDCl_3$ using a Varian XL-100 spectrometer operating at 100.1 MHz. Tetramethylsilane was used as the internal reference standard. IR spectra were recorded in a Beckman spectophotometer.

RESULTS

Polyisoprenoid alcohols were isolated from the seeds of a number of plant species (Table 1). The material obtained from dicotyledonous species could be resolved by HPLC into a family of symmetrical peaks representing alcohols containing from 14 to 22 isoprene units. The same fraction from cereals, however, yielded on HPLC a family of peaks that were nonsymmetrical and appeared to be made up of nearly equal amounts of 2 components (Fig. 2a). This split peak material contained from 13

TABLE 1

Polyisoprenoid Alcohol Content of Seeds[a]

Source	Type of peaks	Concentration (mg/100 g)	Homologues detected	Major homologues
Monocotyledons				
Rye	split	7.7 ± 0.7	13-18	15,16
Maize	split	6.7 ± 0.9	14-18	15,16
Barley	split	6.0 ± 1.2	13-17	14,15
Wheat	split	4.4 ± 0.6	13-17	15,16
Rice[b]	split	1.2 ± 0.2	15-18	16,17
Dicotyledons				
Rapeseed	single	15.9 ± 0.8	14-18	16,17
Peanut	single	9.6 ± 0.7	14-19	15,16
Soybean	single	8.6 ± 0.5	15-22	17,18
Navy bean	single	2.5 ± 0.08	16-20	17,18
Mung bean	single	1.4 ± 0.18	16-20	17,18
Peas	single	1.1 ± 0.07	15-18	16,17

[a]Average ± SD for 4 determinations.
[b]Polished rice.

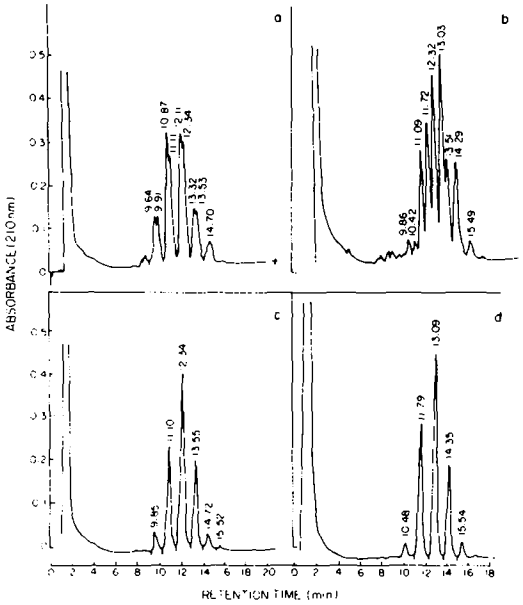

FIG. 2. Reverse-phase HPLC of: (a) wheat germ polyisoprenoid alcohols; (b) wheat germ polyisoprenoid alcohols after MnO_2 oxidation; (c) component I from oxidation mixture (b), after TLC; (d) component II from oxidation mixture (b) after TLC. The figures above individual peaks indicate the retention times for the particular homologue(s) involved. For example, the figures 11.10, 12.34 and 13.55 indicated in panel (c) correspond to retention times for dolichols containing 15, 16 and 17 isoprene units, respectively.

to 18 isoprene units. A similar split peak could be reproduced by mixing equal amounts of dolichol-18 and polyprenol-18, and injecting the mixture into the HPLC. This suggested that the peaks obtained with monocotyledons represented a mixture of dolichols and polyprenols. Wheat germ proved to be a rich source of such material (18 mg/100 g) and was used as the source of the fairly large amounts (100 mg) of polyisoprenoid alcohols required for chemical characterization.

By taking advantage of the known sensitivity of polyprenols to oxidation by MnO_2 (10), wheat germ could be shown to contain both dolichols and polyprenols. Experiments with authentic HPLC standards indicated that only ca. 5% of dolichol is oxidized under conditions that result in complete oxidation of polyprenols to the corresponding aldehydes. Before oxidation, the polyisoprenoid alcohol fraction from wheat germ ran as a single spot on TLC plates of Silica Gel 60H developed in hexane/diethyl ether/acetic acid (65:35:1, v/v/v) (Fig. 3, lane F). After oxidation, 2 spots were obtained, one of which ran with the same R_f

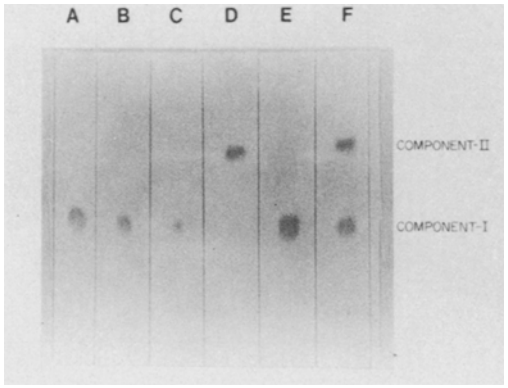

FIG. 3. Thin layer chromatography (TLC) of: (a) human dolichol; (b) human dolichol after MnO_2 oxidation; (c) polyprenol-18; (d) polyprenol-18 after MnO_2 oxidation; (e) wheat germ polyisoprenoid alcohols; (f) wheat germ polyisoprenoid alcohols after MnO_2 oxidation. Small aliquots of standards or test samples were spotted on Silica Gel 60H plates and run in a solvent system of hexane/diethyl ether/acetic acid (65:35:1, v/v/v). Compounds were visualized by exposure to iodine vapor.

(0.34) as dolichol (component I), and the other with the R_f of polyprenol aldehyde (component II, Fig. 3, lane F).

Component I had HPLC retention times that were identical to those of the homologues of dolichol (Fig. 2a and 2c), and also gave an NMR spectrum that was very similar to that of standard dolichol. Component II (presumably polyprenol aldehyde) could be resolved into a family of symmetrical peaks containing from 13 to 18 isoprene units by HPLC (Fig. 2b and 2d). When examined by IR spectroscopy (Fig. 4), component II was shown to possess a C:O stretch at 5.97 μ, which is indicative of a double bond α,β to the aldehyde group (3). By comparison, the corresponding C:O stretch in the aldehyde form of dolichol occurs at 5.78 μ (3). These results indicate that all the cereals examined contain both polyprenols and dolichols, and that the 2 forms encompass the same size range.

The concentration of polyisoprenoid alcohols present in seeds of various plant species varied from 1-16 mg/100 g (Table 1). The values for monocotyledons represent the sum of dolichol and polyprenol forms present.

DISCUSSION

Previous studies in our labortory (8) showed that soybeans contain substantial amounts of dolichols ranging in length from 15 to 22 isoprene units. Results of the present experiments indicate that seeds of other dicotyledonous

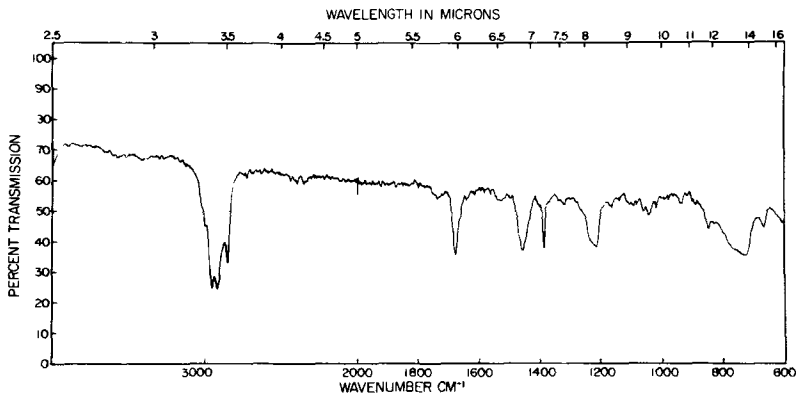

FIG. 4. IR spectrum of component II from TLC.

plants also contain dolichols (Table 1). Although the concentrations were generally higher in oil-rich seeds, no direct relationship to total lipid content was found (11).

In contrast, wheat germ was found to contain approximately equal quantities of dolichols and polyprenols ranging in length from 13 to 18 isoprene units (Fig. 2a). This pattern appeared to be typical of monocotyledonous plants (Table 1).

The reason for this difference is not known. The biosynthesis of dolichols is thought to proceed by a pathway leading first to polyprenyl pyrophosphates, followed by saturation of the α-residue to give dolichyl pyrophosphates that are subsequently dephosphorylated to free dolichol (12). The presence of free polyprenols in monocotyledons may mean that dephosphorylation without saturation of the α-isoprene unit occurs more readily in these species.

Two reports (13,14) have suggested, on the basis of indirect evidence, that dolichol intermediates are involved in the biosynthesis of asparagine-linked glycoproteins in germinating seeds. These intermediates are normally phosphorylated forms of dolichol. Only traces of Dol-P were found in our analysis of soybeans (8), but the concentration may increase during germination. This active form might be required for the biosynthesis of hydrolytic enzymes, which act on the food reserves of the seed. Some of these hydrolases may carry a carbohydrate moiety N-linked to asparagine (15). The polyprenols in monocotyledons may serve a metabolic function different from that of the dolichols.

ACKNOWLEDGMENTS

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REFERENCES

1. Hemming, F.W. (1974) in Biochemistry of Lipids (Goodwin, T.W. ed.), MTP Int. Rev. Sci., Biochemistry Series One, Vol. 4, pp. 39-97, Butterworths, London.
2. Elbein, A.D. (1979) Annu. Rev. Plant Physiol. 30, 239-272.
3. Burgos, J., Hemming, F.W., Pennock, J.F., and Morton, R.A. (1963) Biochem. J. 88, 470-482.
4. Parodi, A.J., and Leloir, L.F. (1979) Biochim. Biophys. Acta 559, 1-37.
5. Ibata, K., Mizuno, M., Takigawa, T., and Tanaka, Y. (1983) Biochem. J. 213, 305-311.
6. Radomińska-Pyrek, A., Chojnacki, T., and Pyrek, J.S. (1979) Biochem. Biophys. Res. Commun. 86, 395-401.
7. Hayes, G.R., and Lucas, J.J. (1980) J. Biol. Chem. 255, 7536-7539.
8. Ravi, K., Rip, J.W., and Carroll, K.K. (1983) Biochem. J. 213, 513-518.
9. Carroll, K.K. (1976) in Lipid Chromatographic Analysis (Marinetti, G.V., ed.) 2nd Ed., Vol. 1, pp. 173-214, Marcel Dekker, Inc., New York.
10. Attenburrow, J., Cameron, A.F.B., Chapman, J.H., Evans, R.M., Hems, B.A., Jansen, A.B.A., and Walker, T. (1952) J. Chem. Soc. 1094-1111.
11. Watt, B.K., and Merrill, A.L. (1963) Composition of Foods, Agriculture Handbook No. 8, U.S. Government Printing Office, Washington, DC.
12. Vigo, C., and Adair, W.L., Jr. (1982) Biosc. Rep. 2, 835-840.
13. Brett, C.T., and Leloir, L.F. (1977) Biochem. J. 161, 93-101.
14. Delmer, D.P., Kulow, C., and Ericson, M.C. (1978) Plant Physiol. 61, 25-29.
15. Akazawa, T., and Miyata, S. (1982) in Essays in Biochem. (Campbell, P.N., and Marshall, R.D., eds.) Vol. 18, pp. 41-78, Academic Press, London.

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Effect of External Abdominal Irradiation on the Dimensions and Characteristics of the Barriers to Passive Transport in the Rat Intestine

A.B.R. THOMSON, C.I. CHEESEMAN and **K. WALKER**, *Departments of Physiology, Medicine and Laboratory Medicine, Division of Gastroenterology, University of Alberta and University of Alberta Hospital, 8-104 Clinical Sciences Bldg., Edmonton, Canada T6G 2G3*

ABSTRACT

Limited information is available on the effect of irradiation on the intestinal absorption of passively transported nutrients. In this study a previously validated in vitro technique was used to measure the uptake of fatty acids (FA), fatty alcohols and cholesterol into the jejunum, ileum and colon of control rats and animals exposed to cesium^{-137} source irradiation applied to the abdomen. The effective resistance of the intestinal unstirred water layer was measured with lauryl alcohol, and in control rats this resistance was lowest in the ileum, highest in the colon, and of intermediate value in the jejunum. Fourteen days after 600 rads, unstirred layer resistance was reduced by half in the colon when the bulk phase was stirred at 600 rpm, and in the jejunum, ileum and colon when the bulk phase was unstirred (0 rpm). The incremental change in the free energy of transfer ($\int\Delta Fw\rightarrow\ell$) was measured with a homologous series of saturated medium-chain length fatty acids; 14 days after 600 and 900 rads the value of $\int\Delta Fw\rightarrow\ell$ in the jejunum rose significantly, and occurred when light and electron microscopic changes were minimal. Fourteen days after 300 rads, the uptake of FA 6:0-12:0 was reduced, but this decline in uptake appeared to be caused by a fall in the functional surface area of the membrane rather than a change in $\int\Delta Fw\rightarrow\ell$. The uptake of cholesterol into the jejunum, ileum and colon was unaffected by irradiation, suggesting that cholesterol and fatty acids may have different diffusion pathways through the membrane. Thus, external abdominal irradiation influences the dimensions and characteristics of the barriers to passive transport in the intestine of the rat, and thereby modifies the uptake of some but not all passively absorbed nutrients. These functional changes are not closely associated with morphological alterations.
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INTRODUCTION

Radiotherapy for malignant human neoplasms is a relatively safe and effectife form of treatment, but its use may become limited by its undesirable side effects on the gastrointestinal tract (1-4). Although changes in the absorption of some nutrients have been described following radiotherapy (5-13), little information is available on the mechanism of the effect of radiation on intestinal transport of passively absorbed solutes. The intestinal uptake of passively transported nutrients is determined by the dimensions and characteristics of 2 diffusion barriers in series, the unstirred layer and the brush border membrane (3). Accordingly, a previously validated in vitro technique was used to examine the uptake of a homologous series of saturated fatty acids (FA), fatty alcohols and cholesterol into the jejunum, ileum and colon of rats exposed to varying doses of cesium^{-137} source irradiation applied externally to the abdomen.

METHODS AND MATERIALS

Chemicals

Unlabeled and [1-^{14}C]-labeled FA and fatty alcohols were all greater than 99% pure as supplied by Applied Sciences Laboratories Inc., State College, PA, and by Sigma Chemical Corp., St. Louis, MO; unlabeled taurodeoxycholic acid was from Sigma, and [1-^{14}C]-cholesterol and [G-^{3}]-dextran were obtained from New England Nuclear, Boston, MA. The radiolabeled dextran had an approximate molecular weight of 15,000-17,000 and was used as a nonpermanent marker of the adherent mucosal volume. All other compounds were of reagent grade and were obtained from Fisher Scientific Co. Ltd., Fairlawn, NJ. The FA probe molecules that were used included acetic, butyric, hexanoic (caproic), octanoic (caprylic), decanoic (capric) and dodecanoic (lauric) acid; the abbreviations used for each of these FA were FA 2:0, FA 4:0, FA 6:0, FA 8:0, FA 10:0 and FA 12:0. The fatty alcohols that were used included hexanol, octanol, decanol and dodecanol (lauryl alcohol).

Preparation of Incubation Solutions

An appropriate amount of both ^{14}C-labeled and unlabeled cholesterol was dissolved in an exact volume of chloroform/methanol (2:1, v/v) in an incubation beaker, and the chloroform/methanol phase was then evaporated to ensure complete removal of the organic solids. Seventy-five ml of a 40 mM taurodeoxycholate

solution in Krebs-bicarbonate buffer (with calcium omitted) were added to the beaker and the solution was stirred with a magnetic bar for 2 hr. The solution was then further diluted by the addition of 75 ml of Krebs-bicarbonate buffer to give a final volume of 150 ml and a final taurodeoxycholate concentration of 20 mM. The final bulk phase concentration of cholesterol was 0.1 mM. The breaker was gassed with 5% carbon dioxide in oxygen for 2 hr at 37 C and, if necessary, the pH was readjusted to 7.4. A trace amount of radiolabeled volume marker [G-^{3}H]-dextran was then added and the solution was ready to be used for the determination of uptake rates of tissues. This technique for preparing micellar incubation solutions has been previously published (14).

The technique used for preparing the test solutions containing FA and fatty alcohols has also been published elsewhere (15-17). Briefly, an appropriate amount of both a ^{14}C-labeled and unlabeled probe molecule were dissolved in 150 ml Krebs-bicarbonate buffer to yield final concentrations of 5 mM for acetic and butyric acid, 1 mM for hexanoic acid, 0.5 mM for decanoic acid and 0.2 mM for lauric acid. The solutions containing the fatty alcohols were prepared in a similar manner and yielded final concentrations of 1 mM for hexanol and octanol, 0.5 mM for decanol and 0.2 mM for lauryl alcohol. These FA and fatty alcohol concentrations were selected to ensure solubility of the fatty probes in the aqueous solution. Previous validation studies have confirmed that the rate of uptake of these probes is linear between 4-12 min incubation, and that a linear relationship existed between concentration and the uptake of FA or fatty alcohol. The counting activity of the solutions was ca. 100,000 cpm/ml of ^{14}C-labeled compounds and 250,000 cpm/ml of ^{3}H-dextran; the nonabsorbable ^{3}H-dextran marker was used in all experiments.

Tissue Preparation

Albino Wistar rats were anesthetized with Nembutal. As is outlined in detail elsewhere (15-17), a short segment of proximal jejunum, distal ileum and transverse colon was rapidly removed, rinsed with 50 ml of cold saline, opened along its mesentery border and the mucosal surface was carefully washed with a stream of cold saline from a syringe to remove visible mucus and debris. Circular discs of intestine were cut from a segment, mounted as a flat sheet in incubation chambers and clamped between 2 plastic plates so that the mucosal and serosal surfaces were exposed to separate incubation solutions, with apertures in the plates exactly 0.25 cm in diameter. To the serosal compartment was added 1.0 ml of Krebs-bicarbonate buffer, and the chambers were transferred to beakers containing oxygenated Krebs-bicarbonate buffer at 37 C for a preincubation period of 30 min. The chambers were then transferred to other beakers for specific experiments.

The preincubation and incubation solutions were mixed at identical stirring rates with circular magnetic bars and the stirring rates were precisely adjusted by means of a strobe light. Stirring rates were reported as the revolutions per minute (rpm) at which the stirring bar was driven. Stirring rates were altered in a systematic and reproducible manner to yield different values of the effective thickness and surface area of the unstirred water layer.

Determination of Rates of Uptake of Fatty Acids and Cholesterol

After preincubation in Krebs-bicarbonate buffer for 30 min, the chambers were transferred to other beakers containing ^{3}H-dextran and various concentrations of ^{14}C-labeled fatty acid or cholesterol in oxygenated Krebs-bicarbonate buffer at 37 C. After incubation for 6 min, the experiment was terminated by removing the chamber and quickly rinsing the tissue in cold saline for ca. 5 sec. The exposed mucosal tissue was then cut out of the chamber with a circular steel punch, blotted on filter paper and placed in a tared counting vial. The tissue was dried overnight in an oven at 75 C and the dry weight was determined. The sample was then saponified with 0.75 N NaOH, scintillation fluid was added and radioactivity was determined by means of an external standardization technique to correct for variable quenching of the 2 isotopes (11,17,18). The rate of uptake, J_d, was calculated after correcting the total tissue ^{14}C-radioactivity for the mass of the probe molecule present in the adherent mucosal fluid; these uptake rates were expressed as the nanomoles of the probe molecule taken up into the mucosa per minute per 100 mg dry weight of tissue. When comparisons were made of the relative uptake rates of the different fatty acids in the homologous series, J_d was expressed as nmol/100 mg/min/mM in order to correct for the varying concentrations of FA in the bulk phase.

Determination of Rates of Uptake of Fatty Alcohols

The method for measuring the rate of uptake of the fatty alcohols was similar to that used for the FA, except that the intestinal tissue was weighed wet and was placed in 1 ml Protosol^R (New England Nuclear Corp.) at 56 C for 18 hr. The samples were cooled to

room temperature (16,17), scintillation fluid was added and radioactivity was determined as described above. Separate pieces of jejunum were weighed wet and reweighed after drying overnight at 75 C. From the ratio of intestinal dry to wet weight, the rate of uptake of the fatty alcohols changed from nmol/min/100 mg wet weight to nmol/min/100 mg dry weight. These latter units are similar to those used for the FA and cholesterol. When comparisons were made of the relative uptake rates of the different fatty alcohols, J_d was expressed as nmol/min/100 mg/mM to correct for the varying concentrations of fatty alcohols in the bulk phase.

In one group of experiments, the bulk phase was stirred at 600 rpm and the rate of uptake of FA 6:0-12:0 was determined after a 6 min incubation period. The natural logarithm of the rate of uptake (nmol/100 mg/min/mM) divided by the appropriate aqueous diffusion coefficient ($\ell n\ J_d/D$) was plotted against the number of carbons in the fatty alcohol. Note that $\ell n\ J_d/D$ is an expression of permeability and is not a flux term. Since the value $\ell n\ J_d/D$ of the fatty alcohols reached a plateau between 10:0 and 12:0, the rate of uptake of lauryl alcohol was determined at different rates of stirring of the bulk phase.

Measurement of Effective Resistance of Intestinal Unstirred Water Layer

The rate of uptake of fatty alcohols 10:0 and 12:0 has previously been shown to be limited by the effective resistance of the unstirred water layer (15,19). This study has also demonstrated that the uptake of these fatty alcohols was limited by diffusion across the unstirred water layers, and therefore the rate of uptake of lauryl alcohol at different stirring rates may be used to estimate the effective resistance of the unstirred layer (16,17,19).

Expression of Results

The rate of uptake, J_d, was calculated after correcting the total tissue ^{14}C radioactivity for the mass of the probe molecule present in the adherent mucosal fluid. The value of the adherent mucosal fluid volume was not influenced by radiation. The uptake rates were expressed as the nmol of the probe molecule taken up into the mucosa per min per 100 mg dry weight of tissue (nmol/min/100 mg). When comparisons were made of the relative uptake rates of the different FA and fatty alcohols in the homologous series, J_d was expressed as nmol/100 mg/min/mM to correct for the varying concentrations of FA in the bulk phase. The values obtained for the different groups are reported as mean ± SEM of the results of 8-12 animals. The statistical significance of the difference between any 2 means was determined using Student's *t* test.

Irradiation of Animals

Animals were allowed ad libitum access to food and water until the morning they were to be irradiated. All animals were anesthetized with Nembutal. One half of the rats were allowed to awaken without being irradiated. While the other rats were asleep, they were placed in a uniform position under a cesium^{-137} source, the head and thorax were shielded and the abdomen was exposed to 300, 600 or 900 rads. Irradiation was applied at a rate of 150 rads/min. The animals were placed on a restrainer that permitted uniform density with shielding of the thorax. Half the dose was applied with the animal supine and half with the animal prone. The animals were then allowed to awaken. The control and irradiated animals were returned to their cages and were again allowed ad libitum access to food and drink until the morning of the absorption studies. The absorption studies were performed 3, 7 and 14 days after external abdominal irradiation.

These doses of irradiation were chosen in an attempt to identify the doses that would permit survival of the animal, yet would also allow the demonstration of functional changes in the intestine. These time intervals of 3, 7 and 14 days were chosen to demonstrate early absorption changes when the animals were not eating, as well as later transport changes when the animals were eating and gaining weight normally, and when the morphology of the intestine was normal.

Parameters of Intestinal Structure

The weight of the full thickness of the intestinal wall exposed to a fixed 0.25 cm diameter aperture in the transport chamber was determined. The ratio of dry/wet intestinal weight was established from the weight of samples of intestine weighed before and after drying overnight. The adherent mucosal fluid volume was determined from the ^{3}H-dextran space.

At the time of laparotomy to obtain intestinal tissue for the absorption studies, full-thickness samples of jejunum and ileum were removed, mounted on mesh and fixed in either Bouin's solution for later staining in H & E for examination by light microscopy, or in glutaraldehyde for subsequent examination by electron microscopy. The micrographs were coded

and read without knowledge of the treatment given. The height and width of the villi were measured from a minimum of 6 samples from each of 4 animals in each group (control, 300, 600 and 900 rads irradiated 14 days earlier). The surface area of the mucosal membrane was calculated (5). The number of cells per villus and the number of villi per unit of serosal length were assessed.

For transmission electron microscopy, specimens were trimmed to 1 x 3 cm and fixed for 4 hr in 3% glutaraldehyde in phosphate buffer at pH 7.2. Afterward, they were rinsed in the above buffer, then fixed for 1 hr in 1% osmium tetroxide in phosphate buffer. The specimens were then dehydrated in ethanol and embedded in epoxy resin. Thin sections were cut and mounted onto copper grids, stained with uranyl acetate and lead citrate, then examined and photographed with a Siemens Elmiskop 102. The height of the microvilli were measured for the columnar cells at the tip of the villus.

RESULTS

Characteristics of Animals

All animals survived the 300 and 600 rads abdominal irradiation, but one-third of the rats perished within 2 days of a single dose of 900 rads. For the first 2 days following irradiation the animals ate less than controls, and by day 3 after irradiation the body weight of the animals irradiated with 600 rads was less than the body weight of the control animals (190 ± 17 g vs 236 ± 19 g, respectively, $p < 0.05$) and the weight of the jejunum per unit of serosal area was also reduced (1.2 ± 0.08 mg vs 1.9 ± 0.06 mg, respectively, $p < 0.05$). The irradiated animals then began to eat normally and by day 7 the jejunal weights of the irradiated and control animals were similar (1.8 ± 0.07 mg vs 1.9 ± 0.06 mg, respectively), even though the body weight of the irradiated animals remained less than controls (191 ± 17 g vs 239 ± 20 g, respectively, $p < 0.05$). The intestinal weights remained similar in the control and irradiated groups at later periods following irradiation. By day 14 the body weights of the control and irradiated groups were similar (270 ± 22 g vs 255 ± 20 g, respectively), and the food intakes in the 2 groups remained similar.

Alcohols

The bulk phase was stirred at 600 rpm to reduce the effective thickness of the unstirred water layer, and the rate of uptake of a homologous series of saturated fatty alcohols was determined in the jejunum, ileum and colon. In control animals, when the chain length of the fatty alcohols was plotted against ℓn J_d/D (natural logarithm of rate of uptake divided by the appropriate aqueous diffusion coefficient of the fatty alcohols), the value of ℓn J_d/D initially rose as the chain length increased from 6 to 10 carbon atoms, but a plateau was reached between alcohols with 10 and 12 carbon atoms (Fig. 1). A similar curvilinear relationship was noted for controls and animals irradiated for 14 days (600 rads) in the jejunum and ileum.

The rate of uptake of lauryl alcohol (12:0) was used to measure the effective resistance of the unstirred water layer (17). When the bulk phase was stirred at 600 rpm to reduce the effective resistance of the unstirred layer, the effective resistance was similar in the jejunum of control and irradiated animals (Fig. 2). The effective resistance was lower in the ileum than in the jejunum, 17.3 ± 1.5 min vs 22.4 ± 2.5 min · 199 mg/cm^3, respectively ($p < 0.05$, and once again no difference was found in unstirred layer resistance in the ileum of control and irradiated animals. When the bulk phase was stirred at 600 rpm, unstirred layer resistance was much higher in the colon than in the jejunum or ileum of control rats, but unstirred layer resistance was lower ($p < 0.05$) in the colon of irradiated than control animals (Fig. 2).

When the bulk phase was unstirred (0 rpm), the effective resistance in the control animals was much higher in each site (Fig. 2). Abdominal irradiation was associated with a decline in unstirred layer resistance in the jejunum from

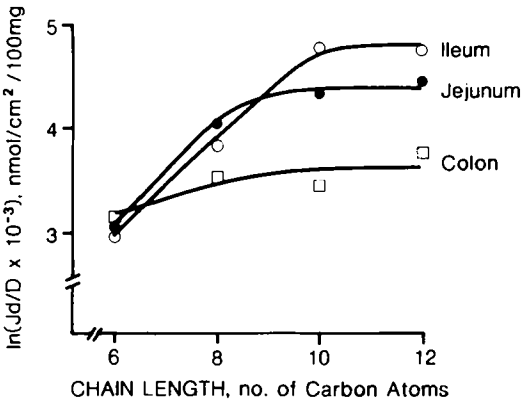

FIG. 1. Rate of uptake of a homologous series of fatty alcohols into the jejunum, ileum and colon of control rats. The bulk phase was stirred at 600 rpm to reduce the effective resistance of the unstirred water layer. Each point represents the mean of the results of 9-12 animals. The size of the SEM was usually smaller than the size of the symbol used to depict the magnitude of the mean.

42.2 ± 3. min to 20.0 ± 1.3 min • 100 mg/cm^3 ($p < 0.05$), and in the ileum from 34.6 ± 2.7 min to 17.6 ± 1.2 min • 100 mg/cm^3 ($p < 0.05$). Similarly, when the bulk phase was unstirred, irradiation was also associated with a lower unstirred layer resistance over the colon (Fig. 2).

Fatty Acids and Cholesterol

Three days after the application of 600 rads externally to the abdomen, the uptake of FA 4:0 and FA 12:0 into the jejunum was significantly increased (Fig. 3). The enhanced uptake of these FA then gradually declined at days 7 and 14. Whereas the uptake of FA 4:0 was less than control values at day 14 ($p < 0.05$), the uptake of FA 12:0 remained above the control value ($p < 0.05$). The uptake of cholesterol into the jejunum was unaffected by external abdominal irradiation.

When the chain length of the homologous series of saturated short- and medium-chain length FA was plotted against $\ell n\, J_d/D$ (natural logarithm of rate of uptake/aqueous diffusion coefficient), a linear relationship was noted between FA 4:0-12:0 in the control (Fig. 4A), and in the rats irradiated for 14 days, and between FA 8:0-12:0 in therats irradiated for 3 days (Fig 4B). This relationship was noted both when the results were expressed as nmol/min/100 mg (Fig. 4A), and as nmol/min (not shown). The slope of this linear component was used to calculate the apparent incremental change in free energy ($\int \Delta Fw \rightarrow \ell$); this value rose from −267 cal/mol in control rats to −400 cal/mol in the rats irradiated for 3 days, and rose above controls to −300 cal/mol in the animals irradiated for 14 days ($p < 0.05$). The values for the rates of uptake shown in Figure 4 were

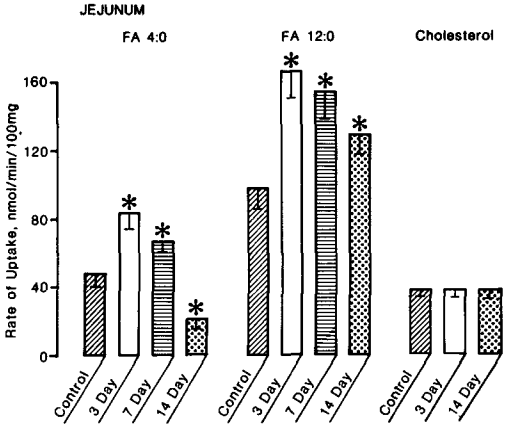

FIG. 3. Rate of uptake of butyric (FA 4:0) and lauric (FA 12:0) acid and cholesterol into the jejunum of control and irradiated rats. The bulk phase was stirred at 600 rpm to reduce the effective resistance of the unstirred water layer. The animals were exposed to 600 rads from a cesium 137 source 3, 7 or 14 days previously. An asterisk (*) indicates that the difference between the mean value of the irradiated and the control groups was statistically significant, $p < 0.05$.

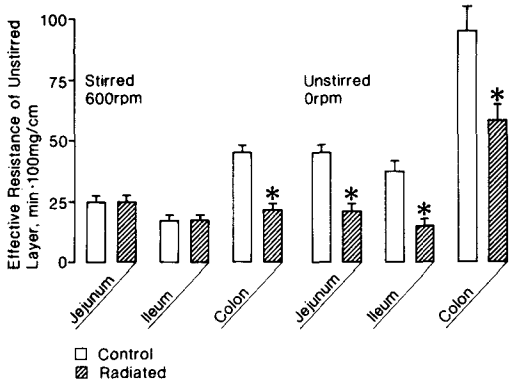

FIG. 2. Effective resistance of the unstirred water layer in control and irradiated rats. The bulk phase was either stirred at 600 rpm, or was unstirred (0 rpm), and the rate of uptake of lauryl alcohol was used to assess the effective resistance of the unstirred layer overlaying the jejunum, ileum and colon. The irradiated animals had been exposed to 600 rads from a cesium 137 source 14 days previously. An asterisk (*) indicates that the difference between the mean value of the irradiated and the control groups was statistically significant, $p < 0.05$.

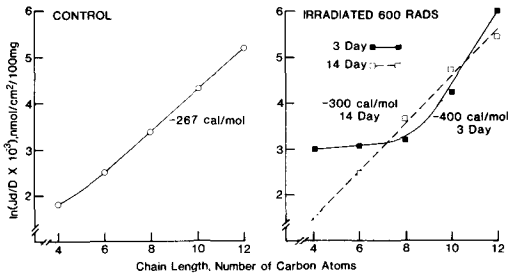

FIG. 4. Rate of uptake of a homologous series of saturated FA into the jejunum of control and irradiated rats. The bulk phase was stirred at 600 rpm to reduce the effective resistance of the unstirred layer. The irradiated animals had been exposed to 600 rads from a cesium 137 source 3 or 14 days previously. Each point represents the mean of the results of 9-12 animals. The size of the SEM was usually smaller than the size of the symbol used to depict the magnitude of the mean. The results are expressed as the natural logarithm of J_d/D when J_d is the rate of uptake of the FA, and D is the free aqueous diffusion coefficient.

uncorrected for the effective resistance of the unstirred layer, and even when the bulk phase was stirred at 600 rpm to reduce unstirred layer resistance, a significant diffusion barrier persisted (Fig. 2). When the rates of uptake of the FA were corrected for unstirred layer resistance, then the rates of uptake were higher, and the incremental change in free energy calculated taking unstirred layer resistance into account was also higher: $\int\Delta Fw\rightarrow\ell$ in the jejunum of control rats was −291 cal/mol, and $\int\Delta Fw\rightarrow\ell$ was −329 cal/mol in the animals irradiated for 14 days ($p < 0.05$).

The uptake of short-chain FA and cholesterol into the ileum and colon was also affected by irradiation of the abdomen. In the ileum the uptake of FA 2:0 was reduced ($p < 0.05$) at days 3 and 14, but the uptake of FA 6:0 and cholesterol was unchanged (Fig. 5). In the colon, the uptake of FA 2.0 and FA 6.0 was significantly reduced 3 and 14 days after irradiation but cholesterol uptake was unaffected (Fig. 6).

Influence of Dose of Irradiation

Fourteen days after the application of 300 rads to the abdomen, a decline occurred in the uptake of FA 6:0-12:0 in the jejunum and colon ($p < 0.05$), but not in the ileum. When the rates of uptake of the FA were corrected for unstirred layer effects and were plotted as $\ln J_d/D$ vs FA chain length, a linear relationship was obtained (Fig. 7). In both the jejunum and the colon, the lines were parallel for controls and animals irradiated with 300 rad, but were lower ($p < 0.05$) in the irradiated than in the control rats. In contrast to the lower rate of uptake of FA into the jejunum and colon 14 days after 300 rads, 14 days after the application of 900 rads to the abdomen, the uptake of

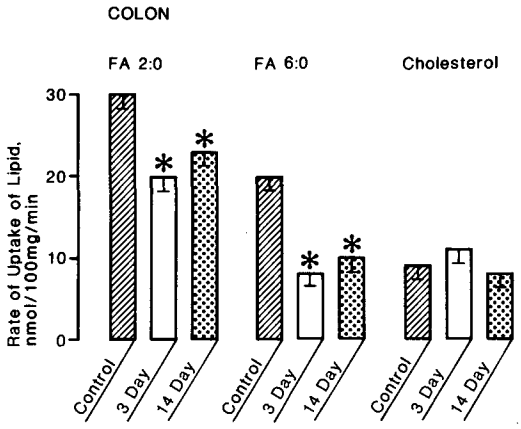

FIG. 6. Rate of uptake of acetic (FA 2:0) and hexanoic (FA 6:0) acid and cholesterol into the colon of control and irradiated rats. The bulk phase was stirred at 600 rpm to reduce the effective resistance of the unstirred water layer. The animals were exposed to 600 rads from a cesium 137 source 3 or 14 days previously. An asterisk (*) indicates that the difference between the mean value of the irradiated and the control groups was statistically significant, $p < 0.05$.

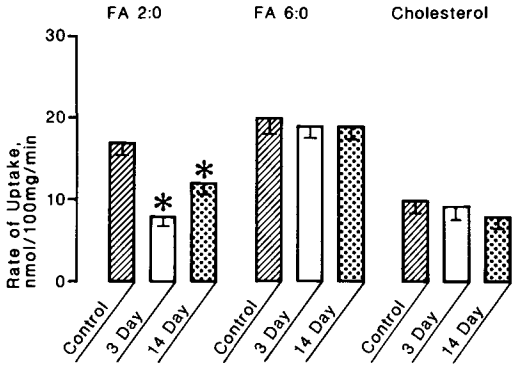

FIG. 5. Rate of uptake of acetic (FA 2:0) and hexanoic (FA 6:0) acid and cholesterol into the ileum of control and irradiated rats. The bulk phase was stirred at 600 rpm to reduce the effective resistance of the unstirred water layer. The animals were exposed to 600 rads from a cesium 137 source 3 or 14 days previously. An asterisk (*) indicates that the difference between the mean value of the irradiated and the control groups was statistically significant, $p < 0.05$.

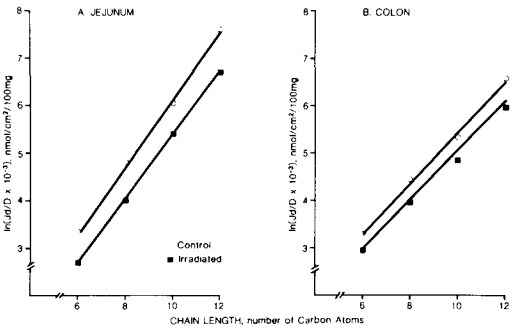

FIG. 7. Rate of uptake of a homologous series of saturated FA into the jejunum and colon of control and irradiated rats. The bulk phase was stirred at 600 rpm to reduce the effective resistance of the unstirred layer. The irradiated animals had been exposed to 300 rads from a cesium 137 source 14 days previously. Each point represents the mean of the results of 9-12 animals. The size of the SEM was usually smaller than the size of the symbol used to depict the magnitude of the mean.

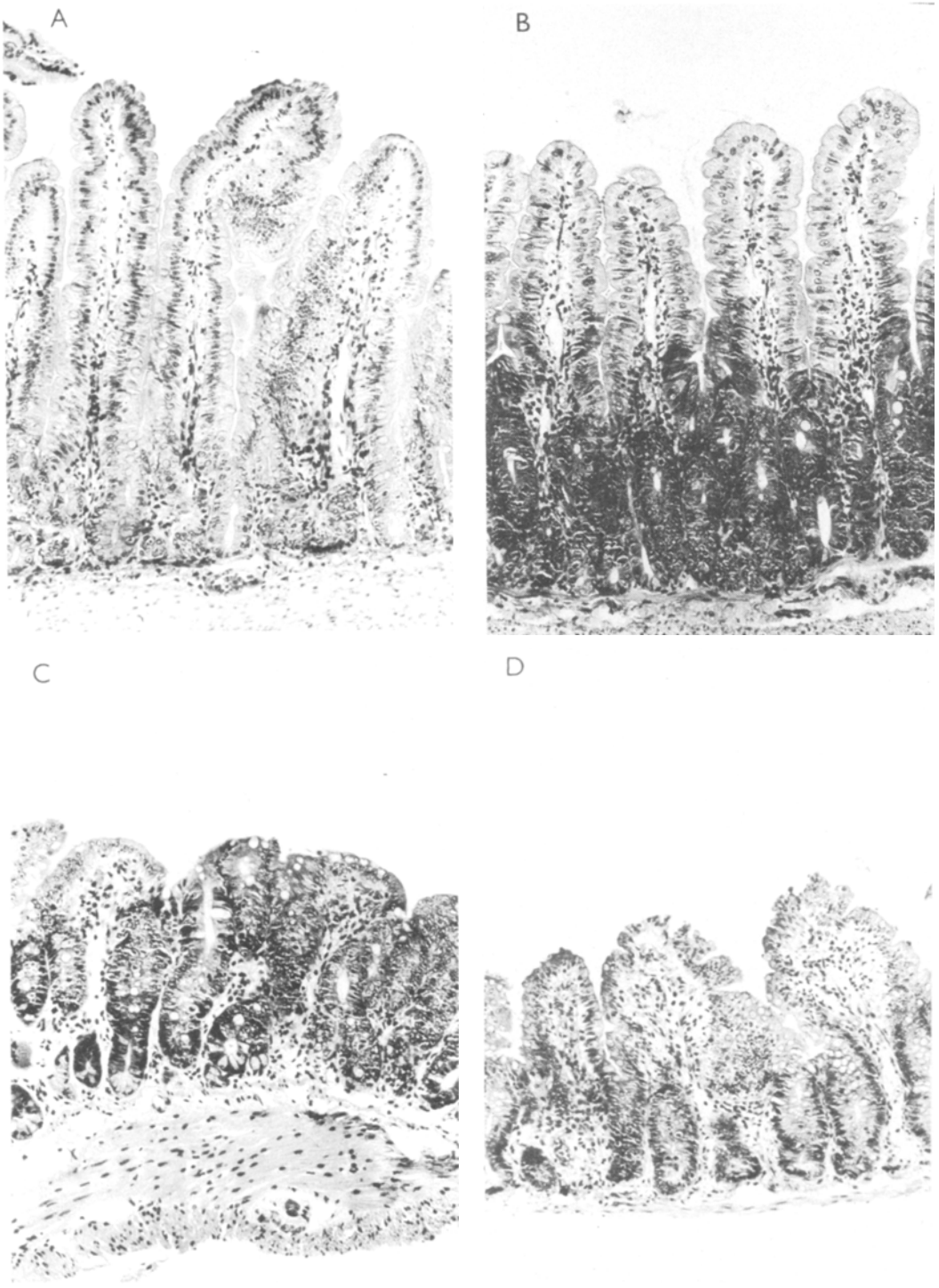

FIG. 8. Light microscopy of irradiated intestine. The tissue was fixed in Bouin's solution, and was stained with H & E. The panels represent (a) jejunum, 600 rads, day 1; (b) jejunum, 600 rads, day 3; (c) ileum, 600 rads, day 3; (d) ileum, 600 rads, day 7; (e) jejunum, 300 rads, day 14; (f) ileum, 900 rads, day 14. The details of the findings are given in the Results section. Magnification × 10. (continued)

FIG. 8 (continued). Light microscopy of irradiated intestine. The tissue was fixed in Bouin's solution, and was stained with H & E. The panels represent (a) jejunum, 600 rads, day 1; (b) jejunum, 600 rads, day 3; (c) ileum, 600 rads, day 3; (d) ileum, 600 rads, day 7; (e) jejunum, 300 rads, day 14; (f) ileum, 900 rads, day 14. The details of the findings are given in the Results section. Magnification × 10.

FA 6:0-12:0 was higher in the irradiated than in the control animals and $\int \Delta Fw \rightarrow \ell$ was higher (not shown).

Intestinal Morphology

One day after the application of 600 rads irradiation to the abdomen, light microscopy of the jejunum and ileum was normal (Fig. 8A). By day 3, deeper crypts developed with active mitoses, and expansion of the regeneration zone with mitoses increased 2/3 of the way up the villus (Fig. 8B); the size and shape of the villi and appearance of the enterocytes remained normal. Similar changes were noted in the ileum, but in addition to the expanded area of regeneration in the crypts, the villi were shorter and the nuclei were enlarged (Fig. 8C). By day 7 after 600 rads of abdominal irradiation, the jejunal villi remained well preserved with only a few interepithelial lymphocytes; by day 14 the jejunum was of normal appearance by light microscopy, although the villi appeared higher. In contrast, at day 7 the ileal villi remained shorter and wider, with enlarged nuclei, loss of nuclear polarity and increased interepithelial lymphocytes (Fig. 8D). By day 14 the villi of the ileum were normal, and only minimal abnormalities persisted in the enterocytes.

At day 14, after 300 rads of abdominal irradiation, a patchy lesion was noted in the jejunum, with a mild abnormality consisting of increased mitotic figures in the crypts extending up the lower third of the villus, blunting and broadening of the villi, enlargement of the nuclei of the enterocytes and loss of basal polarity of the nuclei (Fig. 8E). The lesion in the ileum at 14 days, after 300 rads, was worse than in the jejunum and had many similarities with the ileal lesion 14 days after 600 rads: slight blunting of the villi, interepithelial lymphocyte infiltration, enterocyte absnormalities and increased goblet cells; mitosis less marked than in the ileum at earlier periods after 600 rads (Fig. 8C-D). At 14 days, after 900 rads, the crypts of the jejunum and ileum appeared normal; the villus height was reduced in the ileum (Fig. 8F) but normal in the jejunum. Mild lymphangiectasia was noted at the tip of the villi of the jejunum but not the ileum. These morphological changes were consistent over 10-in. lengths of jejunum and ileum.

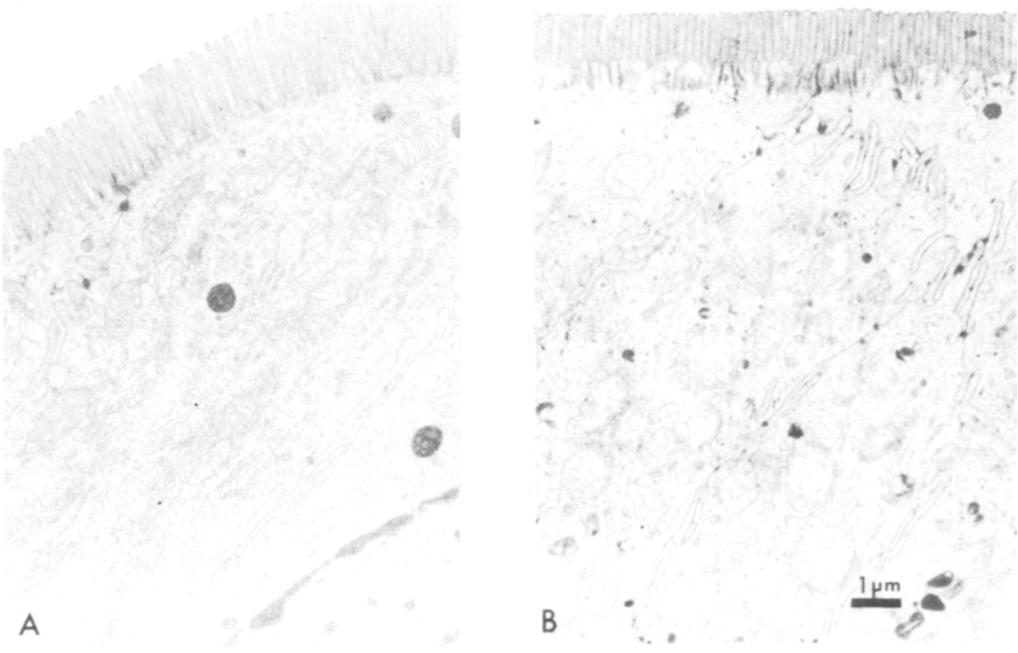

FIG. 9. Electron microscopy of irradiated intestine. The electron photomicrograph depicts an enterocyte near the tip of the jejunal villus from a control rat (A) and an animal exposed to 300 rads abdominal irradiation 14 days previously (B). Magnification × 9000.

At day 14, after 300 rads, the abnormality in the enterocytes of the jejunum and ileum seen on electron microscopy included mild blunting of the microvilli, ballooning of the mitochondria and increasing convolutions of the lateral columnar cell membrane (Fig. 9). The change in the height of the microvilli was influenced by the intestinal site, and by the interval after irradiation, as well as by the dose of radiation (Table 1).

The height and width of the villi, the depth of the crypts, and the surface area of the villi 14 days after 300, 600 and 900 rads are shown in Table 1. In control animals the villi and microvilli were shorter in the ileum than in the jejunum, and the surface area of the ileum was lower in the jejunum than in the ileum ($p < 0.05$). Following abdominal radiation, changes were found in the height of the villi, depth of the crypts and mucosal surface area (Table 1). Fourteen days after abdonominal radiation, the height of the jejunal villi was decreased after 300 rads, increased after 600 rads and unchanged after 900 rads. In contrast, the height of the ileal villi were unchanged after 300 and 600 rads but was increased after 900 rads. The depth of the crypts was reduced in the ileum 14 days after 300 rads and in the jejunum after 600 rads. The surface area of the jejunal mucosa was decreased ca. 25% after 300 rads and increased by the same percentage after 600 rads, whereas the ileal surface area was unchanged after 300 and 600 rads but increased after 900 rads. The number of jejunal mucosal cells per villus was increased after 300, 600 and 900 rads. Thus, the pattern of morphological changes in the intestine differed for the jejunum and ileum and was influenced by the dose of abdominal radiation.

The timing and direction of changes in the microvilli varied with site and did not necessarily coincide with the changes in the villi. For example, in the jejunum the height of the microvilli was greater than in controls 14 days after 600 rads of abdominal radiation, whereas in the ileum the mean height of the microvilli was less than in controls 14 days after 600 rads ($p < 0.05$). By day 14, after 600 rads, the morphology of the villi ahd returned to normal (Fig. 8), the height of the villi and microvilli were increased (Table 1), but the uptake of alcohols, FA and cholesterol was variably affected (Fig. 1-5).

DISCUSSION

The rate of passive transport of nutrients into the body is determined by the dimensions

TABLE 1

Effect of Abdominal Radiation on Dimensions of Villus and Crypt

	14 days after abdominal radiation							
	Controls		300 Rads		600 Rads		900 Rads	
	J	I	J	I	J	I	J	I
Villus height, μm	432 ± 63	222 ± 36	368 ± 82[a]	244 ± 26	523 ± 100[a]	215 ± 50	390 ± 82	290 ± 65[a]
Crypt depth, μm	108 ± 29	103 ± 31	88 ± 35	66 ± 16[a]	71 ± 13[a]	74 ± 26	103 ± 41	85 ± 18
Villus surface area, mm^2	0.466 ± 0.073	0.218 ± 0.039	0.379 ± 0.075[a]	0.260 ± 0.037	0.546 ± 0.075[a]	0.232 ± 0.059	0.440 ± 0.074	0.294 ± 0.052[a]
Number of cells per villus	131 ± 10	63 ± 12	132 ± 29	96 ± 18[a]	181 ± 28[a]	84 ± 15[a]	141 ± 26	118 ± 41[a]
Number of villi per unit serosal length	10.7 ± 2.1	10.9 ± 1.8	8.2 ± 2.1[a]	8.8 ± 2.7	9.7 ± 2.6	13.7 ± 3.6	8.8 ± 1.7[a]	9.7 ± 1.7
Height of microvillus, μm	1.15 ± .01	1.00 ± .01	1.05 ± .01[a]	0.75 ± .01[a]	1.30 ± .01[a]	0.80 ± .01[a]	1.30 ± .01[a]	1.00 ± .01[a]

[a]$p < 0.05$, radiated vs control. The dimensions were obtained for the jejunum (J) and ileum (I). Data expressed as mean ± SEM.

and characteristics of two major barriers in series, the intestinal unstirred water layer and the permeability properties of the brush border membrane (19). The effective resistance of the unstirred water layer is altered according to site along the intestine, with aging and with diabetes mellitus (19). This study has also demonstrated low unstirred layer resistance in the ileum, high resistance in the colon and intermediate values in the jejunum (Fig. 1-2). The magnitude of the effect of irradiation on the unstirred layer resistance depended upon the site and rate of stirring of the bulk phase (Fig. 2). The dimensions of the unstirred layer in vivo are likely closest to those observed in vitro when the bulk phase is unstirred (19), and it is of considerable interest that under these conditions of high resistance (0 rpm), irradiation was associated with a 45% reduction in unstirred layer resistance in jejunum, ileum and colon (Fig. 2). The mechanisms responsible for these changes in unstirred layer resistance with irradiation are unknown, and why the dimensions of this diffusion barrier vary along the intestine is also unknown. At 14 days, after 600 rads, variable changes occurred in the histology of the intestine (Table 1 and Fig. 8). The alterations in unstirred layer resistance following abdominal irradiation are unlikely to be related simply to variations in mucosal morphology. As the mucosal permeability to passive uptake increased 14 days after 600 and 900 rads, we anticipated that the effective resistance of the intestinal layer would decline. This decline in unstirred layer resistance was demonstrated when the bulk phase was unstirred, but was not demonstrated when the bulk phase was stirred at 600 rpm (Fig. 2). The lack of demonstrable effect of abdominal radiation on the unstirred layer resistance over the jejunum when the bulk phase was stirred is probably related to the low value of this resistance at 600 rpm, and to the relatively small change in the value of $\int\Delta Fw\rightarrow\ell$. Finally, when the unstirred layer resistance was high (0 rpm), demonstrating this effect of irradiation in decreasing unstirred layer resistance was possible (Fig. 2).

The permeability properties of a membrane may be best appreciated from the measurement of the incremental change in free energy associated with the uptake of a homologous series of solutes (20,21). Fourteen days following irradiation, the value of $\int\Delta Fw\rightarrow\ell$ increased ($p < 0.05$) from −291 cal/mol in control animals to −329 cal/mol in the rats irradiated at 600 rads after 14 days. The value of $\int\Delta Fw\rightarrow\ell$ also increased 14 days after 900 rads but was unchanged after 300 rads. Thus, irradiation was associated with an increase in the passive permeability properties of the jejunum, but only after moderately high doses of irradiation. Furthermore this result was associated with a decrease in the effective resistance of the unstirred layer when the bulk phase was unstirred and resistance was high. The permeability properties of the intestine 14 days after irradiation were also influenced by the dose: after 300 rads, the uptake of FA declined (Fig. 7), whereas uptake rose after 600 and 900 rads (Fig. 3-4). However, after 300 rads, the value of the incremental change in free energy was unchanged, but the y-axis intercept was lower (Fig. 7). This suggests a lower functional surface area of the jejunal membrane 14 days after 300 rads (22). This suggestion was substantiated by the light microscopic studies, which showed shortening of the jejunal villi 14 days after 300 rads (Table 1). In contrast, the higher value of $\int\Delta Fw\rightarrow\ell$ in the jejunum at day 14 after 600 rads was noted in animals whose jejunal villi were higher than normal. Fourteen days after 900 rads the intestinal structure had also returned to normal, but the incremental change in free energy was increased above the values obtained in either controls or animals exposed to 600 rads. Thus the greater values of $\int\Delta Fw\rightarrow\ell$ could not be related to simple structural changes, and are probably associated with true changes in the permeability properties of the brush border membrane.

We anticipated that morphological changes would be more pronounced after 900 rads. However, morphology was done only at one point in time, 14 days after irradiation. Mucosal damage may have been initially greater after 600 or 900 rads, but that sufficient time had elapsed for apparently normal mucosa to be observed by day 14. This time-course of morphological changes following external abdominal irradiation must now be explored using varying doses of irradiation. However, whether the same proportion or total surface area of the villus is used for passive permeation of each of the lipids studied is unclear. Thus, whether changes in the total surface area of the membrane associated with abdominal radiation (Table 1) is necessarily associated with changes in that portion of the membrane responsible for uptake of the various lipid probes is uncertain.

Despite these changes in the dimensions and characteristics of the unstirred layer and the villus and microvillus membrane, the uptake of some passively transported solutes was unaffected by irradiation: cholesterol uptake into the jejunum, ileum and colon was unchanged (Fig. 3, 5 and 6), and the uptake of FA 6:0 was reduced into the colon (Fig. 6) but not into the

jejunum or ileum (Fig. 4-5). The effect of irradiation also depended on the interval following treatment: the uptake of FA 2:0 into the ileum and colon was reduced 3 and 14 days after irradiation (Fig. 5-6), whereas the uptake of FA 2:0 into the jejunum was reduced at 14 days but was unchanged 3 days following irradiation (Fig. 4). These findings indicate that although irradiation alters the dimensions and characteristics of the barriers to passive transport in the intestine of the rat, the uptake of some but not all passively absorbed nutrients is modified, suggesting that FA and cholesterol may have different diffusion pathways through the membrane.

Three days following irradiation, the animals reduced their intake of food and had failed to gain weight at the same rate as the control rats. By the 14th day after irradiation the rats had comparable body weights and intestinal weights as the control animals, thus the observed transport abnormalities were unlikely to have been related to food intake or intestinal weight. The height of the villi and the mucosal surface area was variable: these values were reduced after 300 rads, increased after 600 rads and normal 14 days after 900 rads, whereas for the ileum, the villus height and mucosal surface area were unchanged except after 900 rads (Table 1). Prolonged and repeated exposure to sublethal doses of total body irradiation induces significant morphologic changes in the mucosa of the small intestine of laboratory animals (7,10,21, 23-26) and man (25,27,28). The increased mitosis in the intestinal crypts, loss of nuclear polarity, interepithelial lymphocyte infiltration and decreased height of the villi quickly reverse following radiotherapy, leaving only subtle abnormalities in brush border enzymes or in scanning electron microscopy (29-33). In this study, no distinguishable difference was found between control and intestine irradiated by 900 rads when examined by light microscopy at 14 days. By electron microscopy performed 14 days after 600 and 900 rads, a slight increase occurred in the height of the microvilli in the jejunum, but decrease in the ileum at 14 days after 300 and 600 rads (Table 1). However, the functional changes persisted, even 14 days after a dose as low as 300 rads (Fig. 7), at which time the height of the microvilli was normal. At 14 days, after 600 rads, for example, alcohol uptake was similar in the jejunum and ileum (Fig. 1-2), even though mocosal surface area was increased in the jejunum and decreased in the ileum (Table 1). Despite the greater surface area in the jejunum 14 days after 600 rads, the uptake of FA 4:0 was reduced, and the uptake of cholesterol was unchanged (Fig. 3). In contrast, in the ileum, the uptake of FA 2:0 was reduced but the uptake of FA 6:0 and cholesterol were unchanged (Fig. 5), despite a greater mucosal surface area (Table 1). These functional changes were also unrelated to the number of mucosal cells per villus. Therefore, this lack of correlation between functional and morphological changes suggests that the changes in the function were related to subtle changes in the diffusion properties of the membrane, rather than being related to changes in the morphology of the villi or microvilli.

Impaired fat absorption has been described in patients undergoing conventional X-ray therapy, Co^{60} teletherapy and the intrauterine application of radium (8-10). The measurement of fat absorption may be a more sensitive indicator of changes occurring in the gastrointestinal tract because of ionizing radiation than are barium studies (5). This might appear to be surprising in light of the findings of this study, because the lower effective resistance of the unstirred layer in the jejunum or ileum of the irradiated animals when the bulk phase was unstirred (Fig. 2) would be expected to be associated with a greater rather than a lesser efficiency of of fat absorption. However, the application of low doses of irradiation to the abdomen (300 rads) was associated with a decline in the uptake of FA into the jejunal and colonic membrane (Fig. 7), rather than an increase in uptake as was found with higher doses of 600 and 900 rads (Fig. 3-6). Thus the dose and possibly the frequency of abdominal irradiation will probably influence the clinical usefulness of fat absorption as a measure of radiation-induced functional damage to the intestine. Furthermore, the collection of stools represents a cumbersome and time-consuming investigation, and developing a simpler and more sensitive clinical tool for the detection of the presence of radiation damage to the intestine is necessary. From the present study, we would predict that the probes that would best reflect the functional changes in the small and large intestine following abdonominal irradiation would include lauryl alcohol (Fig. 2) and medium-chain length FA (Fig. 3-5). Whether these functional changes persist for periods in excess of 14 days, and whether functional changes in the intestine also occur following multiple exposure to low doses of abdominal irradiation remain to be established.

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APPENDIX

Theoretical Considerations

As outlined by Diamond and Wright in their comprehensive analysis of the determinants of cell membrane permeation (20,21), the variations of permeability rates among the different passively absorbed FA can be explained in terms of intermolecular forces that determine solute–water and solute–lipid interactions. A summary of these theoretical considerations has previously been published (16,22,34). Under conditions where the effective resistance of the unstirred layer is low and where membrane permeation is limited by the rate of diffusion through the membrane, then

$$P = e^{-\Delta Fw \rightarrow \ell / RT} \cdot \frac{Dm}{dm} \qquad [1]$$

where P is the true permeability coefficient, Dm is the diffusion coefficient of the probe in the membrane, dm is the effective thickness of the cell membrane, $\int \Delta Fw \rightarrow \ell$ is the free energy change in transferring 1 nmol of FA from the bulk phase to the lipid membrane, R is the gas content and T is the absolute temperature. The apparent passive permeability coefficient, P_d*, of each FA was calculated from

$$P_d^* = J_d / C_1 \qquad [2]$$

where C_1 is the concentration of the probe molecule in the bulk phase. However, these values needed to be corrected for unstirred layer effects (17):

$$C_2 = C_1 - J_d \cdot d / Sw \cdot D \qquad [3]$$

where C_2 is the concentration of the probe molecule of the aqueous membrane interface, J_d is the experimentally determined rate of uptake, d is the effective thickness of the unstirred layer, Sw is the effective surface area of the membrane and D is the aqueous free diffusion coefficient. Then, the value of d/Sw was calculated from the uptake of lauryl alcohol. Because the rate of uptake of lauryl alcohol is limited by diffusion through the unstirred layer, its rate of uptake may be used to estimate the effective resistance of the unstirred layer:

$$J_d = (C_2 - C_1)(DSw/d) \qquad [4]$$

where the reciprocal of SwD/d, i.e., d/SwD, equals the effective resistance of the unstirred layer, with the units min·100 mg/cm^3. Then, the value of the true permeability coefficient P_d may be calculated from

$$P_d = J_d / C_2. \qquad [5]$$

Thus, the quantity of $J_d/C_2/D$, or P_d/D will be proportional to $\int \Delta Fw \rightarrow \ell$. The value of $\int \Delta Fw \rightarrow \ell$ cannot be directly determined, but the change in $\int \Delta Fw \rightarrow \ell$, i.e., the incremental free energy of solution, $\int \Delta Fw \rightarrow \ell$, caused by the addition of a $-CH_2-$ functional group, can be calculated from the true passive permeability coefficient P_d of different members of the homologous series of FA (17,20,21):

$$\int \Delta Fw \rightarrow \ell = (RT)\, n \frac{Pd_1}{Pd_2} \qquad [6]$$

where Pd_1 and Pd_2 are the true permeability coefficients of 2 FA differing only in a functional group.

In an attempt to calculate the incremental free energy changes of the different species, $\ell n\ P_d/D$ was plotted against the number (N) of $-CH_2-$ groups in each FA probe molecule (16). The slope of the linear portion of this relationship between FA 8:0 and FA 12:0 was determined to permit comparison of the incremental free energy changes ($\int \Delta Fw \rightarrow \ell$) of the jejunal membranes of the discs. Previously researchers had reported that changes in the slope of the relationship between $\ell n\ J_d/D$ vs (N) represents a change in the permeability properties of the membrane, whereas a parallel displacement of this relationship represents a change in the surface area of the membrane, used for the uptake of the homologous series of saturated FA, without a change in permeability properties of the membrane (16,17,19,22,24). Furthermore, because the uptake of a homologous series of solutes is being compared, relative rather than absolute changes are being compared and this approach is not influenced by alterations in mucosal mass.

REFERENCES

1. Pierce, M. (1948) edited by W. Bloom, McGraw-Hill, New York, p. 502.

2. Sauer, W.G. (1960) J. Iowa St. Med. Soc. 50, 1-7.
3. Van Dongen, J.M., Visser, W.J., Daems, W.T., and Galjaard, H. (1976) Cell Tiss. Res. 174, 183-199.
4. Viner, S.K. (1958) Med. J. Aust. 2, 255-257.
5. Ecknauer, R., Vadakel, T., and Wepler, R. (1982) J. Geront. 37, 151-155.
6. Hamlet, R., Can, K.E., Toner, P.G., and Mias, A.H.W. (1976) Brit. J. Radiol. 49, 624-629.
7. Quastler, H. (1956) Radiat. Res. 4, 303-320.
8. Quastler, H., Bensted, J.P.M., Lamerton, L.F., and Simpson, S.M. (1959) Brit. J. Radiol. 32, 501-511.
9. Reeves, R.J., Cavanaugh, P.J., Sharpe, K.W., Thorpe, W.A., Winkler, C., and Sanders, A.P. (1965) Am. J. Roentgenol. 94, 848-851.
10. Reeves, R.J., Sanders, A.P., Isley, J.K., Jr., Sharpe, K.W., and Baylin, G.J. (1959) Radiology 73, 398-401.
11. Reeves, R.J., Sanders, A.P., Sharpe, K.W., Thorne, W.A., and Isley, J.K., Jr. (1963) Am. J. Roentgenol Rad. Ther. Nucl. Med. 89, 122-126.
12. Sheeban, J.F. (1944) Arch. Path. 37, 297-308.
13. Warren, S. (1942) Arch. Path. 34, 1070-1079.
14. Wellwood, J.M., and Jackson, B.T. (1973) Brit. J. Surg. 60, 814-818.
15. Kedziora, J., Kanski, M., Clieslinska, D., and Rosiek, S. (1976) Radobiol. Radiother. 17, 737-741.
16. Thomson, A.B.R. (1980) Am. J. Physol. 239, 6363-6371.
17. Warren, S., and Friedman, N.B. (1972) Am. J. Path. 18, 499-507.
18. Sallee, V.L., and Dietschy, J.M. (1973) J. Lipid Res. 14, 475-484.
19. Thomson, A.B.R. (1981) in Basic Mechanisms of Gastrointestinal mucosal Cell Cytoprotection, Grossman, M., ed., Wilkin and Wilkin.
20. Diamond, J.M., and Wright, E.M. (1969) Proc. R. Soc. Land. B. Biol. Sci. 172, 273-316.
21. Diamond, J.M., and Wright, E.M. (1969) Ann. Rev. Physiol. 31, 581-646.
22. Thomson, A.B.R. (1980) J. Lipid Res. 21, 1097-1107.
23. Bloom, M.A. (1950) Radiology 55, 104-115.
24. Parshkov, E.M., and Brokskii, R.A. (1975) Bull. Exp. Biol. Med. 80, 1390-1392.
25. Trier, J.S., and Browning, T.H. (1966) J. Clin. Invest. 45, 194-204.
26. Westergaard, H., and Dietschy, J.M. (1974) J. Clin. Invest. 54, 718-732.
27. Goodrich, J.K., and Hickman, B.T. (1962) Am. J. Roentgenol. Rad. Ther. Nucl. Med. 87, 69-75.
28. Sallee, V.L., Wilson, F.A., and Dietschy, J.M. (1972) J. Lipid Res. 13, 184-192.
29. Becciolini, A., Gerber, B.G., and Deros, J. (1977) Acta Radiol. 16, 87-96.
30. Becciolini, A., Gerber, B.G., Buracahi, A., and Deros, J. (1977) Strahlentherapie 153, 485-488.
31. Greenberger, N.J., and Issellsacher, K.J. (1964) Am. J. Med. 36, 450-456.
32. Mohiuddin, M., Tamura, K., and DeMare, P. (1978) Radiation Res. 74, 186-190.
33. Thomson, A.B.R., Cheeseman, D., and Walker, K. (1983) J. Lab. Clin. Med., in press.
34. Strelina, A.V., and Khanson, K.P. (1978) Med. Radiol. 23, 9-18.

ADDITIONAL REFERENCES

1. Lukie, B.E., Westergaard, H., and Dietschy, J.M. (1974) Gastroenterology 67, 652-661.
2. Thomson, A.B.R., Cheeseman, C., and Walker, K. (1983) J. Lab. Clin. Med., in press.
3. Westergaard, H., and Dietschy, J.M. (1976) J. Clin. Invest. 58, 97-108.
4. Wimber, D.R., and Lamerton, L.F. (1963) Radiation Res. 18, 137-152.

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Hepatic Origin of Triglycerides in Fatty Livers Produced by the Continuous Intragastric Infusion of an Ethanol Diet

HIDEKAZU TSUKAMOTO,[a,b] GAIL LEW,[a] EDWARD C. LARKIN,[a,b] COREY LARGMAN[a,b] and G. ANANDA RAO,[a]* [a]*Veterans Administration Medical Center, Martinez, CA 94553 and* [b]*Department of Internal Medicine, University of California, Davis, CA 95616*

ABSTRACT

Male Wistar rats were maintained for 30 days on an independent and continuous intragastric infusion of ethanol and nutritionally defined liquid diet containing only a small amount of corn oil (CO–4.9% calories). Ethanol intake was progressively increased from 32% to 40.4% of the total calories to maintain a high degree of intoxication during this period. Rats in the control group were infused with an isocaloric diet in which alcohol was replaced by dextrose. The liver triglyceride (TG) content of rats given alcohol (61.5 ± 16.4 mg/g) was ca. 10-fold greater than that of controls (5.9 ± 2.1 mg/g) and similar to that observed previously in rats fed an ethanol diet containing high levels of fat (35% and 43% calories). In TG of fatty liver, the level of 18:2 was small (3%), even though CO in the diet contained a high level of this acid. Furthermore, 16:1 and 16:0 contents were markedly elevated (16% and 40%, respectively) despite the fact that CO did not contain 16:1 and had only a small amount of 16:0. Liver TG having a fatty acid (FA) composition markedly different from that of CO and the presence of high levels of 16:1 and 16:0 indicate that the TG accumulated in the fatty liver originated from hepatic lipogenesis rather than from dietary fat.
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INTRODUCTION

The biological effects of ethanol ingestion have been investigated by a variety of experimental procedures. Some studies have used an acute dose of alcohol. In others, alcohol was mixed with either drinking water or liquid diets in order to provide chronic doses of alcohol to animals.

We have recently developed a new model of chronic alcohol intoxication in the rat (1) that involves the continuous intragastric infusion of ethanol and a liquid diet via double gastrotomy cannulas. The advantages of this model compared with those where the ethanol diets are fed ad libitum are: (a) the natural aversion of animals toward diets containing high levels of alcohol can be avoided, therefore, an increasing daily dose of ethanol can be administered; (b) serial blood samples can be collected via a central venous cannula from an unrestrained and undisturbed animal; (c) the ethanol intake can be modulated to maintain the blood alcohol levels; (d) the dietary composition and intake can be maximally controlled.

In earlier studies, severe fatty liver was produced in the rat following chronic ingestion of ethanol with a liquid diet containing high levels of fat (35% and 43% calories) (2-7). A reduction in dietary fat to 25% or less resulted in a significant decrease of hepatic triglyceride accumulation (3). In the present study, however, severe fatty liver similar to that induced with a high fat ethanol diet was observed following the intragastric infusion of ethanol and a diet containing only 4.9% fat. Detailed analysis of the fatty acid (FA) composition of liver lipids suggested that the fatty liver developed mainly because of the accumulation of triglycerides (TG) produced de novo rather than those from dietary fat.

*To whom correspondence should be addressed.

MATERIALS AND METHODS

Animal Model

Details of the model have been described elsewhere, including cannulation procedures, dietary regimen and blood alcohol level achieved (8-10). Male Wistar rats weighing 350-400 g were used. Either single (control group) or double (alcohol group) gastrostomy cannula were surgically implanted as previously described (8). The use of spring coils and swivels allowed the protection of the cannula and the free movement of animals in individual metabolism cages. The low-fat liquid diet originally described by Thompson and Reitz (11) was used. In this diet, protein was provided by lactalbumin hydrolysate, carbohydrate by glucose and fat by corn oil (CO). The liquid diet was infused through 1 lumen of the double gastrostomy cannula at a rate of 120 ml/kg/day. Ethanol was adminstrered through the other lumen of the gastrostomy cannula at a constant rate of 80 ml/kg/day and an increase in dose was achieved by raising the ethanol concentration. Caloric contributions of ethanol and

macronutrients at an ethanol dose level of 8 g/kg/day were: 24.9% of the total calories as protein, 4.9% as fat, 35.8% as carbohydrate and 32.0% as ethanol. The dose of ethanol was progressively increased to 11.5 g/kg/day to maintain a high degree of intoxication over a period of 30 days. The diet and glucose solution (isocaloric to ethanol infused) were mixed and infused to pair-fed controls through single gastrostomy cannulas at a rate of 200 ml/kg/day.

Quantitation and Analysis of Liver Lipids

Following 30 days of alcohol intoxication, rats were sacrificed, and a small piece of liver (300-500 mg) was cut from the central portion of the middle lobe and weighed. Total lipids were extracted from liver and separated into classes by thin layer chromatography (TLC) as described previously (5). The amount of FA methylesters produced from the total lipid, TG, and phospholipid (PL) fractions was determined by gas liquid chromatography using methylpentadecanoate as an internal standard (5).

RESULTS AND DISCUSSION

Fatty Liver

In the livers of rats given a continuous intragastric infusion of ethanol, the level of total lipids was ca. 3-fold greater than that in controls (Table 1). As observed by many investigators (2-7), the increase in the content of total liver lipids is mostly caused by a rise in the TG level. In rats given ethanol, the content of liver TG was ca. 10-fold greater than that in controls (Table 1). Despite feeding a low-fat diet, the hepatic TG content was similar to that found earlier in rats given a liquid diet containing ethanol and the high levels (35% and 43%) of fat (2-7).

The results of the effects of chronic alcohol consumption on the hepatic levels of PL conflict. Although the increased levels of liver lipids in animals fed alcohol are mainly caused by the enhanced TG content, a small but significant rise in PL content has been observed in some studies (5,6). The PL levels in the livers of animals fed alcohol have also been found to decrease compared with the levels in controls (4). In the present study, the liver PL content in rats given alcohol also appeared to be reduced, although not statistically different from the content in the controls (Table 1).

Fatty Acid Composition of Liver Lipids

Although the content of liver lipids in rats infused with ethanol and a low-fat diet in this study was similar to that obtained earlier in animals given a high-fat ethanol diet (5-7), several differences in the FA composition were observed. For example, the levels of 16:0 and 16:1 observed in earlier studies were low (12-16% and 1-2%, respectively) (5-7). However, they were found to be high in the present study (38% and 14%, respectively) (Table 2). Furthermore, the proportions of 18:1 (43-52%) and 18:2 (17-23%) in liver lipids were high in previous experiments (5-7) whereas they were low (29% and 4%, respectively) in this study. Unlike the case with animals fed a high-fat diet and ethanol, the levels of 20:4 were also low, 9% vs 4% (5-7) (Table 2). Although 20:3 was not detected in lipids in earlier experiments, appreciable levels of this acid were found (Table 2). Similar changes in the composition of FA were also observed in the PL (Table 3). These differences may be related to the types and levels of fat fed to the animals in this and earlier studies. A decreased level of 18:2ω6 and the presence of appreciable amounts of 20:3ω9 suggest that CO in the diet did not provide adequate levels of 18:2 to meet the requirement of essential FA. Whether this is caused by an incomplete intestinal absorption of fat during the continuous intragastric infusion of the diet or an inadequacy in the level of CO in the diet is not known at the present time.

Although similar amounts of TG/g liver were

TABLE 1

Lipid Content in the Livers of Rats Given Intragastric Infusion of an Ethanol or Control Diet[a]

Diet	Total lipid	Triglyceride	Phospholipid
Control	27.49 ± 1.37	5.91 ± 2.05	20.82 ± 1.13
Alcohol	83.45 ± 19.13[b]	61.51 ± 16.45[b]	16.85 ± 1.93[c]

[a]The contents of total lipid, triglyceride and phospholipid are given as mg FA methyl esters/g liver. The values given are mean ± SE from separate analysis of liver lipids from 5 rats in each group.
[b]Significantly different ($p<0.001$) compared with controls (2=tailed t-test).
[c]Not significant compared with controls (2=tailed t-test).

TABLE 2

Fatty Acid Composition of Total Lipids from Livers of Rats Given Intragastric Infusion of a control or Alcohol Diet[a]

Fatty acid	Diet	
	Control	Alcohol
14:0	0.7 ± 0.1	1.3 ± 0.04
16:0	23.2 ± 1.6	37.8 ± 6.6
16:1ω7	6.7 ± 0.2	13.7 ± 2.1
18:0	17.2 ± 0.4	7.7 ± 3.2
18:1ω9	19.9 ± 0.7	29.4 ± 0.9
18:2ω6	8.9 ± 1.6	4.4 ± 1.6
20:3ω9	1.2 ± 0.1	0.7 ± 0.4
20:4ω6	19.8 ± 1.0	4.4 ± 2.7
22:6ω3	1.6 ± 0.4	0.7 ± 0.06

[a]Values given are percentage of total FA. These are mean ± SE from separate analysis with liver in each diet group.

found when the animals were given alcohol and a high- (5-7) or low-fat diet (Table 1), the FA compositions were markedly different (Table 4). As in the case of total lipids, the levels of 16:0 and 16:1 were several times greater, and those of 18:1 and 18:2 were much smaller in TG than the levels found in earlier experiments (5-7). Furthermore, detectable levels of 20:4ω6 were not found in TG (Table 4); appreciable levels of this polyenoic acid were observed when rats were fed a high-fat ethanol diet (5-7).

The FA composition of TG in the fatty liver caused by the intragastric infusion of ethanol was also quite different from that of CO in the diet. No detectable levels of 16:1 were found in CO whereas this monoenoic acid was ca. 16% in the TG of fatty liver (Table 4). In CO, 16:0 comprised only ca. 8%, whereas this acid was the major component (40%) of TG in fatty liver. Although 18:2 was the predominant acid (60%) in CO, only a small level of this acid (3%) was present in liver TG (Table 4). These differences in the FA composition between CO and liver TG show that the origin of fatty liver was not dietary fat.

Origin of TG in Fatty Liver

The chronic consumption of alcohol produces fatty liver not only when the diet contains high levels of fat (2-7), but also when fat is not fed (12). When a high-fat ethanol diet is fed, the FA that accumulate in the fatty liver TG are of dietary origin (5,13). Furthermore, the 2-monoglyceride backbone of dietary TG is retained predominantly by the TG of fatty liver (14). Thus, from these observations, we conclude that in animals fed high levels of fat, alcohol-induced fatty liver is caused by the accumulation of dietary fat. On the contrary, when fat is excluded from the diet, hepatic lipogenesis is stimulated (15-17). Hence, the fatty liver produced by the chronic ingestion of an ethanol fat-free diet must be caused by the accumulation of TG synthesized de novo (12). The severe fatty liver found in the present study also appears to be derived from hepatic lipogenesis. This is suggested by the FA composition of hepatic lipids.

Changes in the relative levels of 16:1 to 16:0 in tissue lipids have been considered to indicate alterations in the levels of desaturase activity. In earlier studies, when fatty liver was produced by feeding rats an ethanol diet with high levels of fat, a 16:1/16:0 ratio in total lipids and TG was ca. 0.1 (5). This ratio increased greatly (0.4) in the corresponding lipids of fatty liver in the present study (Tables 2 and 4). Such an increase in the relative level of 16:1 shows that the desaturase activity was enhanced significantly in the fatty livers produced in this study compared with those obtained by feeding an ethanol high-fat diet. The various nutritional conditions that stimulate liver desaturase activity also enhance lipogenesis. Thus, a greater degree of hepatic lipogenesis occurred in rats given an intragastric infusion of alcohol diet than those fed the Lieber-DeCarli ethanol diet in earlier studies.

Unlike the present study, fatty liver was not produced when rats were fed a Lieber-DeCarli alcohol diet containing 5% calories as fat (3). The reason for this discrepancy is not known. Unlike the case of fatty livers produced by the intragastric infusion of alcohol diet, hepatic lipogenesis may have been inhibited in the livers of rats fed the ethanol diet containing 5% calories of fat. The 18:2 levels in the fat were

TABLE 3

Fatty Acid Composition of Phospholipids from Livers of Rats Given Intragastric Infusion of a Control or Alcohol Diet[a]

Fatty acid	Diet	
	Control	Alcohol
14:0	0.4 ± 0.06	0.3 ± 0.07
16:0	19.4 ± 0.9	22.9 ± 1.5
16:1ω7	5.7 ± 0.5	4.9 ± 1.4
18:0	20.6 ± 0.4	23.9 ± 1.1
18:1ω9	14.5 ± 0.2	14.1 ± 0.7
18:2ω6	9.2 ± 0.2	10.0 ± 1.7
20:3ω9	1.5 ± 0.1	2.6 ± 0.8
20:4ω6	25.3 ± 0.3	17.0 ± 1.6
22:6ω3	2.2 ± 0.3	3.6 ± 0.4

[a]Values given are percentage of total FA and are given as mean ± SE.

TABLE 4

Fatty Acid Composition of Triglyceride from Livers of Rats Given Intragastric Infusion of a Control or Alcohol Diet[a]

Fatty acid	Diet	
	Control	Alcohol
14:0	1.3 ± 0.1	2.0 ± 0.5
16:0	30.8 ± 3.7	40.3 ± 2.3
16:1ω7	14.8 ± 0.2	15.7 ± 2.0
18:0	3.2 ± 1.7	2.7 ± 0.9
18:1ω9	41.7 ± 3.7	36.0 ± 4.1
18:2ω6	7.1 ± 3.9	3.0 ± 0.5

[a]Values given are percentage of total FA and are given as mean ± SE.

25% of total acids (5). In addition to the diet fat, rats were fed ethyl linoleate (2% calories) to prevent essential FA deficiency (3). When animals are fed diets containing polyunsaturated fat, hepatic lipogenesis is depressed (15, 18-20).

In the fatty liver produced by chronic ingestion of a fat-free ethanol diet, the level of FA synthetase in cytosol was several times greater than that in the fatty liver produced by feeding a high-fat ethanol diet (21). When rats are given a continuous intragastric infusion of ethanol and a diet containing a small amount of CO, the levels of hepatic FA synthetase are probably also greater than those in rats fed a high-fat alcohol diet ad libitum. Results obtained in the present and earlier (12) studies support the concept that high levels of dietary fat are not an absolute requirement for production of severe fatty liver induced by chronic consumption of alcohol.

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REFERENCES

1. Tsukamoto, H., French, S., Ihrig, T., Sun, P., Reidelberger, R., and Largman, C. (1983) Fed. Proc. 42, 386.
2. DeCarli, L.M., and Lieber, C.S. (1967) J. Nutr. 91, 331-336.
3. Lieber, C.S., and DeCarli, L.M. (1970) Am. J. Clin. Nutr. 23, 474-478.
4. Stanko, R.T., Mendelow, H., Shinozuka, H., and Adibi, S.A. (1978) J. Lab. Clin. Med. 92, 228-235.
5. Goheen, S.C., Larkin, E.C., Manix, M., and Rao, G.A. (1980) Lipids 15, 328-336.
6. Goheen, S.C., Pearson, E.E., Larkin, E.C., and Rao, G.A. (1981) Lipids 16, 43-51.
7. Goheen, S.C., Larkin, E.C., and Rao, G.A. (1981) Subst. Alcohol Action Misuse 2, 311-319.
8. Tsukamoto, H., Reidelberger, R.D., French, S.W., and Largman, C. Am. J. Physiol. (in press).
9. Tsukamoto, H., French, S.W., Benson, N., Rao, G.A., Larkin, E.C., and Largman, C. Hepatology (submitted for publication).
10. Tsukamoto, H., French, S.W., Reidelberger, R.D., and Largman, C., Am. J. Physiol. (submitted for publication).
11. Thompson, J.A., and Reitz, R.C. (1978) Lipids 13, 540-550.
12. Goheen, S.C., Larkin, E.C., and Rao, G.A. (1983) Lipids 18, 285-290.
13. Lieber, C.S., Spritz, N., and DeCarli, L.M. (1966) J. Clin. Invest. 45, 51-62.
14. Rao, G.A., Lew, G., and Larkin, E.C. (1983) Subst. Alcohol Action Misuse 4, 355-359.
15. Wiegand, R.D., Rao, G.A., and Reiser, R. (1973) J. Nutr. 103, 1414-1424.
16. Hill, R., Webster, W.W., Linazosoro, J.M., and Chaikoff, I.L. (1960) J. Lipid Res. 1, 150-153.
17. Triscari, J., Hamilton, J.G., and Sullivan, A.C. (1978) J. Nutr. 108, 815-825.
18. Sabine, J.R., McGrath, H., and Abraham, S. (1969) J. Nutr. 98, 312-318.
19. Clarke, S.D., Romsos, D.R., and Leveille, G.A. (1977) J. Nutr. 107, 1170-1181.
20. Schwartz, R.S., and Abraham, S. (1982) Biochem. Biophys. Acta 711, 316-326.
21. Rao, G.A., Lew, G., and Larkin, E.C. (1984) Lipids 19, 151-153.

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Hydrocarbon Transport in Chylomicrons and High-Density Lipoproteins in Rat

A. VOST and **N. MACLEAN**, *McGill University Medical Clinic, The Montreal General Hospital, Montreal P.Q. H3G 1A4, Canada*

ABSTRACT

A lipoprotein system is described that transports gut hydrocarbons of low polarity in chylomicrons of intestinal lymph and plasma to plasma high density lipoproteins (HDL) in rat. Four highly lipophilic aryl and alkyl hydrocarbons [benzo(α)pyrene; 1,1,1-trichloro-2,2-bis(p-chlorophenol)ethane (DDT), hexadecane and octadecane] were selected to give a graded range of polarity. Chylomicrons were labeled doubly with radioisotopes in triacylglycerol and a single hydrocarbon by feeding [^{3}H]-glycerol and [^{14}C]hydrocarbon. All hydrocarbons were transported in the triacylglycerol oil phase of chylomicrons. Injected chylomicron triacylglycerol and 3 of 4 hydrocarbons were cleared simultaneously from plasma consistent with lipoprotein-lipase dependent hydrocarbon clearance but DDT was cleared more rapidly. HDL was the major plasma acceptor of all labeled hydrocarbons. Plasma chemical fluxes were measured for octadecane and DDT and both showed net fluxes from chylomicrons to HDL. HDL selectively concentrated chylomicron hydrocarbons from chylomicron triacylglycerol. Lipoprotein lipase stimulation by intravenous heparin significantly increased transfer of alkanes from chylomicrons to HDL. These results indicate that (a) chylomicrons transport gut-derived hydrocarbons with a wide range of structure and polarity as triacylglycerol solutes; (b) HDL are a major plasma acceptor of all these hydrocarbons, demonstrating both selective solute uptake from triacylglycerol and net chemical uptake for the 2 hydrocarbons studied and (c) efflux of these chylomicron hydrocarbons from plasma and into HDL is regulated partly by hydrolysis of chylomicron triacylglycerol.

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INTRODUCTION

Lymph chylomicrons are the major transport proteins for long-chain fatty acids, monoacylglycerols and cholesterol absorbed from gut. These lipoproteins also transport minor quantities of lipophilic compounds, some with a physiological function such as vitamins A (1), D (2) and E (3,4) and some without specific functions, e.g., hexadecane (5) and esters of phytol and cetyl alcohols (6). In vivo lymph chylomicron transport from gut has been demonstrated for 2 polycylic aromatic hydrocarbons—the carcinogen, 9,10-dimethyl-1,2 dimethyl-1,2 benzanthracene (DMBA) (7) and 1,1,1-trichloro-2,2-bis (p-chlorophenol) ethane (DDT)—and the subsequent plasma transport of chylomicron DDT has been studied (8). DDT and DMBA are recovered quantitatively from the oil phase of disrupted chylomicrons and other highly lipophilic compounds are probably transported in the central oil core of chylomicrons, which is composed predominantly of triacylglycerols (9).

The purpose of this study was to extend these isolated observations to determine if hydrocarbons with a wide, defined range of polarity and variable molecular structure share a common transport system from gut as solutes in lymph chylomicron core lipid and subsequently in plasma. Following their absorption from gut, the association of these hydrocarbons with chylomicron core lipid was determined. The subsequent transport, in vivo, of chylomicron core hydrocarbons in plasma, the dependence of hydrocarbon plasma clearance on chylomicron triacylglycerol clearance and hydrocarbon transport to other lipoproteins, were examined. The potential importance in solute transport of partially metabolized chylomicrons or chylomicron remnant particles was not studied.

MATERIALS AND METHODS

Chylomicrons

Lymph was obtained from male Wistar rats with thoracic ducts cannulated and duodenums incubated, 2 days previously (8). After an 8 hr fast, labeled hydrocarbons in 300 μl safflower oil (Hain Pure Food Co., Los Angeles, CA) with or without 10-20 mg unlabeled carrier, all emulsified with sodium taurocholate (35 mM) in 0.6 ml saline, were infused for 5 min. One hundred μCi [^{3}H]glycerol in 5 ml saline was infused continuously between 60-90 min after fat feeding. Peak absorption lymph was collected between 30-90 min after fat feeding and stored coagulated under N_2 at 8 C for a maximum of 60 hr. Chylomicrons were prepared by layering lymph under 2 cm of 0.15 M sodium

chloride solution, pH 7.4, in 6 cm tubes centrifuged at 10^6 *g*/min in a swinging bucket (SB 405) in a B-60 ultracentrifuge (International Equipment Co., Needham, MA). The top 2 cm of chylomicrons were removed by slicing the tube, then redispersed and the process repeated. Chylomicrons were used immediately and had the following characteristics. Their triacylglycerol/protein (w/w) range was 18-50. Chylomicron lipid was ^{3}H labeled specifically in glycerol moieties of acylglycerols; following saponification, less than 2% of [^{3}H]lipid was recovered in fatty acids. 90.3 ± 0.8% (n = 4) of [^{3}H]lipid was in triacylglycerols and 5.3 ± 1.7% (n = 3) in diacylglycerols. Hydrocarbon metabolites were minor and [^{14}C] or [^{3}H] octadecane, hexadexacane, benzo(α)pyrene and DDT accounted for 93%, 91%, 90% and 98% of radiochemical lipid in 4 chylomicron collections with each hydrocarbon. Chylomicrons were obtained at high and low concentrations of octadecane and DDT. High concentrations of these solutes (0.5-3% of triacylglycerol weight) were achieved by feeding oil with 2-8% octadecane or DDT.

Male Wistar rats (290-340 g) fed chow ad libitum, were cannulated in the R jugular vein and carotid artery and maintained under light ether anesthesia for 30 min before injection (8). All control animals were matched by weight. Chylomicrons (vol of 0.4-0.8 ml and triacylglycerol concentrations of 7-50 mg/ml) were injected intravenously in 20 sec. Complete plasma mixing required 3-4 min. Serial arterial blood samples were collected in EDTA (1 mg/ml) and all plasma analyses began immediately. Livers for lipid analysis were flushed with 10 ml ice-cold saline via portal veins before and after removal. Initial results of plasma clearance of chylomicron triacylglycerol and [^{14}C]alkane (n = 3) or [^{14}C]DDT (n = 3) showed no differences between nonanesthetized animals in restraining cages and anesthetized rats that were used subsequently. The specificity of ^{3}H for neutral acylglycerols and of ^{14}C for each hydrocarbon in chylomicrons remained high in plasma sampled 10 min or 14 min after chylomicron injection; 95.6 ± 0.85% (n = 6) of plasma [^{3}H]lipid was recovered in tri- and diacylglycerols and 93.4%, 92.4% and 98.5% of [^{14}C]lipid and 90.2% of [^{3}H]lipid was recovered from chromatograms at the R_F of octadecane, hexadecane, DDT and benzopyrene, respectively, in single experiments with each.

Functional hepatectomy was performed under ether anesthesia by ligating superior and inferior mesenteric arteries, the coeliac axis and portal vein and removing gut between esophagus and mid-rectum. Blood glucose concentrations remained above 100 mg/dl after this procedure but animals required 2-3 ml serum after hepatectomy to restore arterial pressure. Chylomicrons were injected in the R jugular vein when animals were fully active, 30 min after abdominal closure.

Materials

Hexadecane, octadecane (Applied Science Labs., Waltham, PA), pentadecane, eicosane (Polyscience Corp., Niles, IL) and DDT (Aldrich Chemical Co., Milwaukee, WI) were specified at greater than 99% purity. (D+) galactosamine hydrochloride was purchased from Sigma Chemical Co., St. Louis, MO; [1-^{14}C]hexadecane (54 mCi/mmol), [1-^{14}C]octadecane (21 mCi/mmol), [6-^{3}H]benzo(α)pyrene (21 Ci/mmol), [1(3)-^{3}H]glycerol (2.4 Ci/mmol) and [^{14}C]palmitic acid (256 mCi/mmol) were specified at greater than 99% purity (Amersham Corp., Oakville, Ontario, Canada). The purity of all labeled solutes was reconfirmed by glass fiber paper chromatography (8).

Analyses

The major lipoprotein classes in plasma were separated by column chromatography with a 90 cm column of agarose gel beads (6% [w/v] agarose A5m, 200-400 mesh, Bio Rad Labs., Richmond, CA) (1) modified by elution with Tris-HCL buffer (0.18M, pH 8.1 with 0.2g EDTA/1). Elution was monitored with a Uvicord detector (254 nm, LKB Produkter, Sweden). Column elution vol of ultracentrifugally prepared very low density (VLDL), low density (LDL) and high density lipoproteins (HDL) (11) from rat were identical to those of major peaks I, II and III in whole plasma and were identical to elution vol reported for human and rabbit lipoproteins (10). Chylomicron [^{3}H]triacylglycerol and [^{14}C]-hydrocarbons were recovered quantitatively from Peak I and no significant ^{3}H or ^{14}C were detected in elution volumes corresponding to Peaks II and III. Column recoveries of ^{3}H and ^{14}C were greater than 88%. Distribution of hydrocarbons among chromatographed plasma lipoproteins was not altered in preliminary experiments by collecting blood in 0.4 mM 5,5′dithiobis (2-nitro-benzoic acid), an inhibitor of lecithin cholesteryl acyltransferase (LCAT) activity, and this was not used subequently. To reduce ultracentrifugation artefact (12,13), whole plasma was chromatographed in place of $d < 1.21$ plasma lipoproteins. Column fractions for electrophoresis were reconcentrated by ultrafiltration (Minicon-B 15 concentrators,

Amicon Corp., Danvers, MA) to reduce lipoprotein damage. When multiple plasma samples were analyzed, lipid radioactivity in HDL was separated from that in less dense lipoproteins by precipitating the latter with Mg^{++} and sodium phosphotungstate (15). HDL previously separated from plasma by other methods were examined for α-mobility by electrophoresis on paper in a Durrum cell with barbital buffer at pH 8.6 and 1% bovine albumin; lipoprotein standards were detected by Oil Red O stain (16). HDL were separated from plasma by 3 methods—column chromatography, ultracentrifugation and Mg^{++} precipitation and results with each method were compared with at least one other method, including electrophoresis. As shown in the Results section, general agreement occurred between methods in quantitative measurement of HDL-labeled hydrocarbon. The oil phase of chylomicrons was obtained by physical disruption (8).

Plasma lipids were extracted in heptane-isopropanol (8). Triacylglycerols were measured by enzymatic dehydrogenation of glycerol (16) and plasma cholesterol by the combined cholesterol esterase-oxidase enzymatic method (17). Chylomicron protein was measured after diethyl ether extraction of lipid turbidity (18). Alkanes were separated from chylomicrons and plasma lipids by saponification and column chromatography of the unsaponified fraction on silicic acid with hexane/diethyl ether (95:5) elution. The eluted alkanes were separated by gas liquid chromatography (GLC) (19) on an 8 ft × 0.125 in. o.d. stainless-steel column with 3% JXR silicone on 80-100 mesh Gas Chrom Q (Chromatographic Specialties, Brockville, Ontario, Canada) in a gas chromatograph (Model 5830A with 1885A terminal, Hewlett-Packard Co., Palo Alto, CA) with temperature programming from 70-200 C at 4 C/min and flame ionization detection (FID). Sample alkane mass was calculated from internal standards of pentadecane and eiosane and recoveries from added [^{14}C]octadecane. Neutral lipids, DDT and its metabolites, were separated by glass fiber paper chromatography with silica gel G or silicic acid (Gelman Sciences, Ann Arbor, MI) (8,20). The purity of [^{14}C] or [^{3}H]alkanes and benzpyrene were examined by chromatography on silicic acid paper developed in 100% isooctane (8,20). ^{3}H and ^{14}C were assayed by simultaneous scintillation spectrometry (8).

The polarity of hydrophobic solutes in lipid was measured by partition in a biphasic system of tetrahydrofuran/cyclohexane/propylene glycol (2:2.6:2, v/v/v). The partition coefficient, Q, is the ratio of solute in the upper nonpolar phase to that in the more polar lower phase. Q for radioisotope-labeled benzpyrene, DDT, decane, hexadecane and octadecane at 0.2 μM in the total system were 2, 5, 9, 24 and 34, respectively, in order of decreasing polarity.

In Vitro Transfer of Hydrocarbons

Chylomicrons containing either [^{14}C]octadecane or [^{14}C]DDT were incubated with serum from fasting rats, individual rat lipoprotein fractions dialyzed against 0.15 M saline or bovine serum albumin (Fraction V, Winley Morris Co., Montreal, Canada), all at pH 7.2 with EDTA. Incubation in polypropylene tubes was at 4 C or 37 C at 100 cycles/min in a metabolic incubator, with rapid cooling to 4 C after incubation. To compare the transfer of hydrocarbons from chylomicrons to different proteins, chylomicrons were separated from them by flotation in centrifuged sucrose gradients as previously described (8) modified by using 25-40% sucrose in an SB 283 swinging bucket rotor and centrifuging for 60 min at 48,000 *g*. In these conditions, chylomicrons are recovered from tubes 4, 5 and 6 (96% chylomicron [^{3}H]acylglycerol) of a 6-tube elution from the bottom of the gradient and LDL, HDL and albumin remain at the origin in tubes 1 and 2. The presence or absence of hydrocarbon transfer to HDL in plasma was also measured by separating HDL from other incubate lipoproteins by Mg^{++} precipitation.

Results are expressed as means ± SEM. Data were analyzed for significance by Student's *t* test. Plasma clearance ($t_{½}$s), time constants (k) and correlation coefficients (r) were obtained from monoexponential decay curves fitted by nonlinear least squares using a programmed microcomputer (Statistician 342, Compucorp, Los Angeles, CA).

RESULTS

The distribution of labeled hydrocarbons between chylomicron apoprotein-phospholipid "membrane" and interior triacylglycerol-rich oil core was studied by comparing $^{3}H/^{14}C$ ratios in intact chylomicrons ($^{3}H/^{14}C = 1.0$) with released core oil. Ratios in core oil were similar to those in intact chylomicrons for octadecane (0.97 and 0.98), hexadecane (0.99), DDT (0.98 and 0.96) and for benzo(α)pyrene (1.02 and 1.05). These results are consistent with quantitative transport of all 4 hydrocarbons in the triacylglycerol phase of chylomicrons.

Plasma [^{3}H]triacylglycerol 50% occupancy times, measured after intravenous pulse injections of double radioisotope-labeled chylomicrons, correlated positively with the mass of

chylomicron triacylglycerol injected per kg body weight ($r = 0.71$, $n = 9$, $P < 0.01$). Plasma 50% occupancy times of [^{3}H]acylglycerols for injected loads greater than 80 mg/kg body weight were 12.5 ± 1.5 min ($n = 3$) and for loads less than 80 mg/kg were 7.5 ± 1 min ($n = 6$).

The simultaneous plasma clearances of hydrocarbons and triacylglycerols from chylomicrons were analyzed for [^{14}C]hexadecane, [^{14}C]octadecane, [^{14}C]DDT, [^{3}H]benzpyrene and triacylglycerol labeled with [^{3}H]glycerol or [^{14}C]palmitic acid. The results for one typical experiment with each hydrocarbon are given in Figure 1.

Hexadecane, octadecane and benzopyrene were cleared from plasma simultaneously with triacylglycerol in the initial 10 min. DDT was cleared more rapidly than triacylglycerol. The fractional clearance from triacylglycerol is shown in Figure 2 for the 4 solutes. Hexadecane, octadecane and benzpyrene were cleared at mean fractional rates that were within 7% of those of [^{3}H]triacylglycerol in the initial 4-10 min. By 14 min, hexadecane clearance exceeded that of triacylglycerol. [^{14}C]DDT was cleared rapidly from plasma [^{3}H]triacylglycerol with first-order kinetics in all experiments. DDT clearance was partly independent of chylomicron triacylglycerol clearance, because complete inhibition of the latter by galactosamine pretreatment (750 mg/kg body weight, intraperitoneally 24 hr previously [22]) failed to block DDT clearance as shown in Figure 3. DDT clearance from triacylglycerol continued at a reduced rate after galactosamine ($t½ = 3.40 \pm 0.01$ min) compared with controls ($t½ = 2.53 \pm 0.16$ min) and normal animals receiving other chylomicron batches ($t½ = 2.55 \pm 0.34$ min, $n = 4$).

The effect of hydrocarbon concentration on initial plasma clearance was examined for hydrocarbons cleared from triacylglycerol either more rapidly (DDT) or simultaneously (octadecane). Mean fractional clearance rates per minute (FCR) of labeled hydrocarbons from plasma [^{3}H]triacylglycerol were compared at 50-fold concentration changes of each hydrocarbon in triacylglycerol (octadecane < 0.02%, $n = 3$, and > 1.00%, $n = 3$, injected at triacylglycerol loads of 53 ± 8 mg/kg body weight; DDT < 0.01%, $n = 4$ and > 0.50%, $n = 3$, injected at triacylglycerol loads of 122 ± 27 and 139 ± 36 mg/kg, respectively). Between 4-10 min, octadecane FCR at low and high octadecane concentrations were −0.019 ± 0.009 and −0.028 ± 0.014. For DDT, between 2 and 6 min, FCR at low and high concentrations of DDT were 0.173 ± 0.022 and 0.195 ± 0.025.

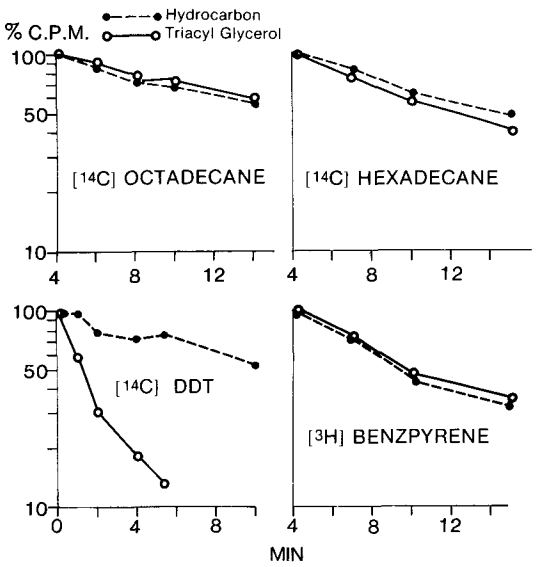

FIG. 1. Chylomicron triacylglycerol and solute clearances from plasma were measured simultaneously in 4 experiments each with either [^{14}C]octadecane, [^{14}C]hexadecane, [^{14}C]DDT or [^{3}H]benzpyrene and either [^{3}H] or [^{14}C]triacylglycerol. Mean triacylglycerol load was 68 ± 25 mg/kg and solute loads/kg were 5.0 μg, 50 μg, 15 μg and 75 ng respectively. Abscissa = min after chylomicron injection.

No effect of concentration on clearance of either hydrocarbon was observed. These results with DDT are consistent with rapid clearance by passive diffusion. Triacylglycerol loads did not correlate significantly with fractional clearance rates for either hydrocarbon.

The liver contained important quantities of [^{3}H] and [^{14}C]hydrocarbons cleared from plasma in random experiments. Liver lipid accounted for 42% and 14% of cleared [^{3}H]-benzpyrene in livers removed 8 and 13 min after chylomicron injection, for 30 ± 3% of [^{14}C]DDT ($n = 6$, time = 14 min) and for 34% and 39% of [^{14}C]hexadecane and [^{14}C]-octadecane, respectively, ($n = 2$) at 14 min. The effect of functional hepatectomy on plasma solute clearance was examined for octadecane and for DDT. In DDT experiments, the t½ values from the initial 2-6 min monoexponential phase of clearance from triacylglycerol (2.93 ± 0.05 min in 3 normal rats, and 4.3 ± 0.05 in 2 controls) was decreased markedly in hepatectomized animals (12.7 ± 2 min, $n = 3$). Serum [^{14}C]octadecane clearance was greatly reduced in relation to [^{3}H]triacylglycerol in hepatectomized animals as shown in Figure 4. [^{14}C]Octadecane was cleared from plasma of hepatectomized animals but from 4 min to 10 min, the t½ of 32 ± 3 min greatly exceeded the t½ in sham-operated controls (7 min and

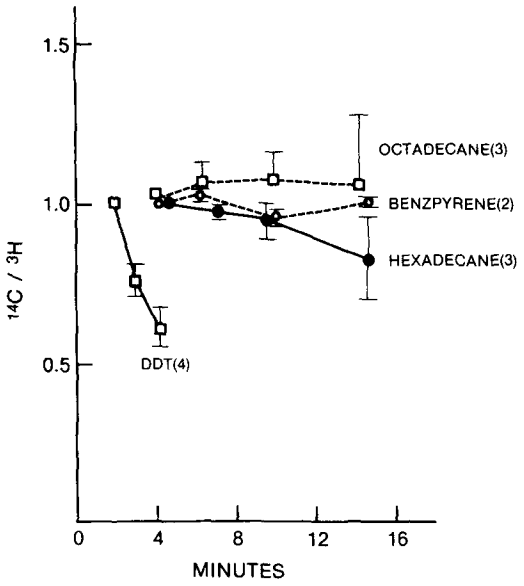

FIG. 2. Fractional clearance of 4 solutes from plasma chylomicron triacylglycerol at varying solutes loads. Results are fractions of initial plasma sample ratio of hydrocarbon to triacylglycerol d.p.m. [^{3}H]-Benzpyrene results are shown as the $^3H/^{14}C$ ratio. For octadecane, hexadecane and DDT, solute loads were, respectively, 5-1500 μg, 6-50 μg and 1-3,900 μg/kg body weight and benzpyrene was used only at low loads of 75 ng/kg. Triacylglycerol loads were 46 ± 6 mg, 102 ± 30 mg, 110 ± 42 mg and 40 ± 18 mg/kg, respectively. Means ± SEM.

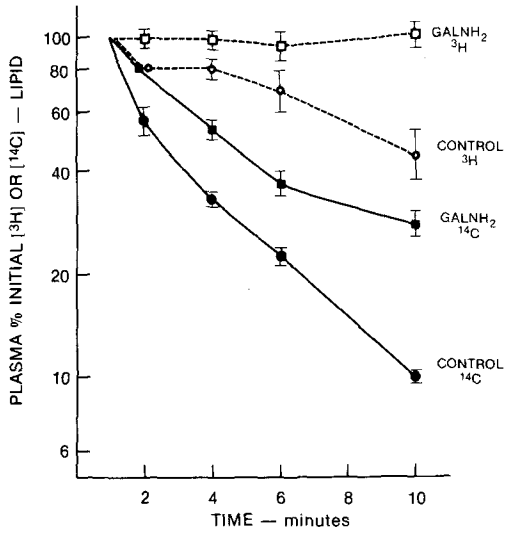

FIG. 3. Plasma clearance of [^{14}C]DDT during galactosamine induced inhibition of [^{3}H]triacylglycerol clearance. Two animals injected with galactosamine were matched with control rats injected with saline. Injected chylomicron loads were 21 mg and 32 mg triacylglycerol and 0.8 mg and 0.6 mg DDT/kg body weight, respectively, for each pair. Means ± range.

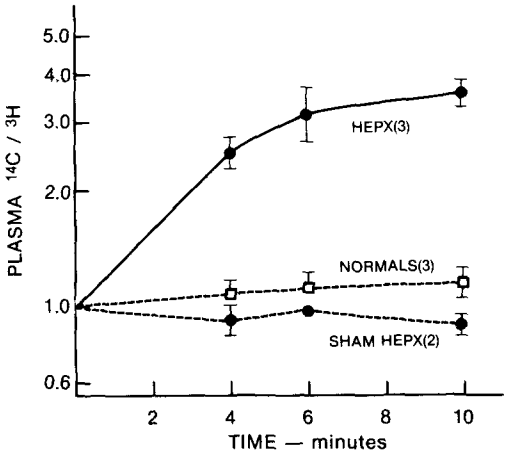

FIG. 4. Effect of functional hepatectomy on fractional clearance of [^{14}C]octadecane from [^{3}H]triacylglycerol. The control animals were sham operated. The injected chylomicron load contained 17 mg/kg body weight triacylglycerol and 14.4 μg/kg octadecane in hepatectomized and control animals and 53 mg/kg triacylglycerol containing 0.005-1% octadecane (w/w) in the normal series. Means ± SEM.

7.6 min) and in normal rats (9.25 ± 2.04 min, $n = 3$, $P < 0.01$). These results indicate that in intact animals, liver was an important site of chylomicron hydrocarbon clearance and that hepatectomy delayed clearance of solutes from plasma and from the triacylglycerol phase of chylomicrons.

To determine if chylomicron hydrocarbons were transported by HDL or lipoproteins other than chylomicrons and their degradation product, chylomicron remnant particles, plasma lipoproteins were analyzed after separation by agarose column chromatography at varying times following injection of chylomicrons containing [^{14}C]hexadecane, [^{14}C]octadecane, [^{14}C]DDT or [^{3}H]benzpyrene. Examples from plasma column chromatograms for each of the 4 hydrocarbons are shown in Figure 5. The major peaks of labeled solute and triacylglycerol were recovered in the leading Peak I of chylomicrons, VLDL and intermediate lipoproteins. The other major labeled hydrocarbon peak eluted with Peak III, plasma HDL, in all experiments. In 8 of 9 experiments, a smaller third hydrocarbon peak eluted with the plasma LDL in Peak II. The distribution of labeled hydrocarbons in plasma for all experiments is given in Table 1. The combined eluate of Peaks II and III (HDL) accounted for 19.7% of labeled solutes in the plasma of the series in Table 2. Seventy percent (range 54-88%) of this

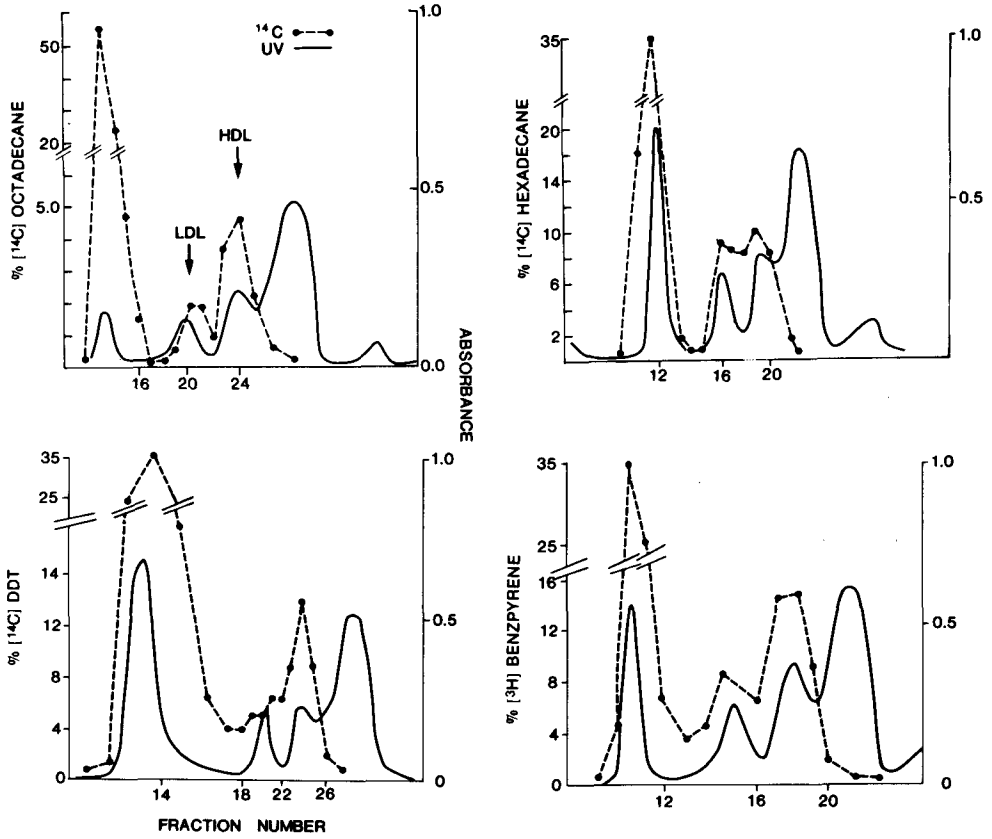

FIG. 5. Plasma chromatograms from agarose bead columns from 4 animals injected with chylomicrons transporting hydrocarbons. Plasma was taken from animals injected with [^{14}C]octadecane, [^{14}C]hexadecane, [^{14}C]DDT and [^{3}H]benzpyrene, respectively, at 30 min, 15 min, 4 min and 15 min after injection; ——, absorbance at 254 nm; -----, percentage of plasma of total labeled hydrocarbon.

TABLE 1

Distribution of Chylomicron-Labeled Solutes in Plasma in Vivo

Solute	Time (min)	Plasma [^{14}C] solute (%) Load (μg)	LDL (%)	HDL (%)	HDL/LDL
Hexadecane	15	<2	1.6	11.9	7.4
Hexadecane	15	<2	17.8	26.0	1.5
Octadecane	10	37	5.4	6.4	1.2
Octadecane	10	10	4.1	5.2	1.3
Octadecane	30	10	4.3	11.5	2.7
DDT	4	100	4.2	5.5	1.3
DDT	4	13	7.9	15.0	1.9
DDT	4	13	8.8	12.0	1.4
Benzopyrene	15	<2	8.6	20.7	2.4

Plasma was obtained at varying times after chylomicron solute injection. Plasma lipoproteins were separated by agarose column chromatography. The loads are expressed as μg solute/ml plasma.

TABLE 2

Chylomicron Octadecane Net Chemical Transport to High Density Lipoproteins in Plasma in Vivo

	μg Octadecane/ml plasma				Chylomicrons Octadecane g / Acylglycerol g
	HDL 0 min	HDL 10 min	Net transport	Octadecane load	
Method					
Mg^{++} ppt	0.32	2.73	+2.41	37	0.034
Ultracentrifuge	0.20	2.05	+1.85	37	0.034
Column	–	(1.56)	–	37	0.034
Ultracentrifuge	0.14	0.88	+0.74	10	0.010
Ultracentrifuge	0.17	0.98	+0.81	10	0.010
Column	–	(0.61)	–	10	0.010
Mean ± SE	0.208 ± 0.046		1.45 ± 0.47		

Plasma HDL chemical and radiochemical octadecane were measured before and 10 min after injection of chylomicrons transporting octadecane. Three methods of HDL preparation were used but the agarose column chromatogram values show only radiochemical transport. Net chemical transport was measured in 4 animals.

material was in Peak III and in all experiments this exceeded Peak II labeled solute ($n = 9$, $P < 0.01$). In 3 experiments labeled solute peaks associated with Peak II and III proteins showed incomplete separation (e.g., [^{14}C]-hexadecane, Figure 5). The minor solute fraction eluted with Peak II was not characterized further. The hydrocarbon-transporting proteins, eluted from gel chromatograms with Peak III (HDL), were concentrated and electrophoresed on paper in 3 alkane and 2 aromatic hydrocarbon experiments. Plasma [^{14}C]DDT radioactivity was insufficient for this analysis. In all experiments, labeled solute was recovered quantitatively with rat serum α-lipoprotein band (benzpyrene, 88% and 89%, hexadecane, 88% and 92%; octadecane, 92%) and no significant radioactivity was recovered in the β band. These experiments demonstrated that hydrocarbon-transporting proteins in Peak III had the hydrated diameter and electrophoretic mobility characteristic of HDL.

Two hydrocarbons, one alkyl and one aromatic, octadecane and DDT, were selected to measure chemical fluxes from chylomicrons to HDL. Because endogenous octadecane was present in rat plasma before chylomicron injection, chylomicron and HDL octadecane concentration and specific radioactivity were measured to calculate fluxes. Significant endogenous DDT was not detected in plasma from control animals and DDT transfers were calculated directly from the specific radioactivity of fed DDT. (Pooled sera from 5 control animals were analyzed by GLC by Dr. J. Scott, Pesticide Research Labs., Dept. of Agriculture, Government of Canada, Ottawa, and DDT content was less than 5 ng/ml.)

Chylomicrons were prepared with octadecane concentrations in triacylglycerol of 1.0% and 3.4% by weight and injected at similar triacylglycerol loads of 38 mg and 43 mg/kg body weight, respectively.

The results from 4 experiments demonstrate a significant net chemical transport of octadecane into the HDL fractions after 10 min of chylomicron metabolism and this flux was doubled in the experiments with higher octadecane concentrations (Table 2). Radiochemical octadecane fluxes were 56% of chemical values and the specific radioactivity of HDL octadecane was consistently less (47-82%) than that of chylomicron octadecane, indicating dilution by endogenous octadecane. The recovered [^{14}C]octadecane percentage distribution in lipoproteins was similar with ultracentrifugal and agarose separations, respectively, yielding 86.5, 2.1, 10 and 1.5 and 86.1, 3.9, 6.7 and 3.2 in VLDL-IDL, LDL, HDL and VHDL.

In similar experiments, chylomicrons containing DDT, at concentrations of 0.5% and 2.0% of triacylglycerol by mass were injected at higher triacylglycerol loads of 110 mg and 190 mg/kg body weight, respectively, to obtain a higher ^{14}C input. The data in Table 3 show net chemical transport of DDT to HDL, measured both by Mg^{++} precipitation and column chromatography. Higher influx to HDL was observed with chylomicrons containing higher solute concentrations. The results demonstrate that 2 lipid solutes with major differences in molecular structure, octadecane and DDT, show net chemical flux into the HDL fractions, with similar quantitative results when HDL is separated by either precipitation or chromatography.

TABLE 3

Chylomicron DDT Net Chemical Transport to High Density Lipoproteins in Plasma in Vivo

	μg DDT/ml plasma				Chylomicrons DDT g
Expt. no.	HDL (0 min)	HDL (4 min)	Net transport	DDT load	Triacylglycerol g
1a	0	1.11	+1.11	100	0.020
1b	0	1.32	+1.32	100	0.020
2	0	0.44	+0.44	27	0.005
3	0	0.49	+0.49	27	0.005
Mean ± SE			+0.84 ± 0.26		

Plasma HDL [^{14}C] DDT was measured 4 min after injection of chylomicrons containing [^{14}C] DDT of known specific radioactivity. HDL was separated from other lipoproteins by either precipitation (experiment 1a) or agarose column chromatography (experiment 1b, 2,3). Chylomicrons were injected in 3 animals.

TABLE 4

Chylomicron Solute and Triacylglycerol Distribution in Serum Lipoproteins in Vivo

	^{14}C/^{3}H				Solute mol Triacylglycerol mol	
		Plasma				
Solute	Chylomicrons injected	Peak I	Peak II	Peak III	Chylomicrons	Peak III
Octadecane	1.0	0.91	5.4	10	0.122	1.22
Octadecane	1.0	1.00	2.5	90	0.035	3.10
DDT	1.0	0.07	3.7	13	0.052	0.68
DDT	1.0	0.19	7.7	25	0.010	0.25
DDT	1.0	0.17	4.0	12	0.010	0.11
Hexadecane	1.0	0.34	1.9	50	–	–
Hexadecane	1.0	–	–	9	–	–
Octadecane	1.0	–	–	21	0.122	2.53

Chylomicrons labeled with [^{3}H] triacylglycerol and [^{14}C] solutes were injected intravenously and subsequent plasma samples were chromatographed on agarose bead columns. The molar ratios of lipids transferred to Peak III (HDL) were calculated from specific radioactivities of chylomicron solute and triacylglycerol. In order, from the table, plasma was sampled at 10 min, 10 min, 4 min, 4 min, 4 min 15 min, 14 min and 12 min after chylomicron injection and triacylglycerol loads were 40 mg/kg, 40 mg/kg, 200 mg/kg, 110 mg/kg, 110 mg/kg, 120 mg/kg, 40 mg/kg and 40 mg/kg, respectively. In the last 2 experiments, HDL was obtained by precipitation.

Dissociation of neutral acylglycerols from hydrocarbon transported from chylomicrons to HDL was calculated from specific radioactivities in chylomicrons. The small quantities of [^{3}H]lipid in HDL were assumed to be neutral acylglycerol and not analyzed for phospholipids, which also transfer from chylomicrons to HDL in rats (23). Consequently the high solute/neutral acylglycerol ratios in HDL in Table 4 are minimal values. In all experiments the Peak I, chylomicron and chylomicron-remnant particle fraction contained 98 ± 0.7% (n = 8) of plasma [^{3}H]lipid and the [^{14}C]solute concentration in [^{3}H]triacylglycerol was either equal to or less than that in injected chylomicrons. In all experiments the HDL fraction ^{14}C/^{3}H and relative molar solute concentration in transferred lipid exceeded that in chylomicrons by 10-90 times ($P < 0.01$). The demonstration of HDL concentration and dissociation of chylomicron radiochemical solute from acylglycerols and the concomitant net chemical transfer of solute from chylomicrons to HDL, suggest that HDL has a specific function in solute removal from chylomicrons.

Plasma kinetics of 3 of the 4 hydrocarbons indicated that plasma hydrocarbon clearance correlated closely with triacylglycerol clearance, a lipoprotein lipase mediated function (24). The transfer of solute to HDL in vivo was studied during stimulaton of the lipoprotein lipase system by intravenous heparin. Heparin (45 USP units/kg) was injected over 20 sec either 1 min or 4 min after the chylomicron

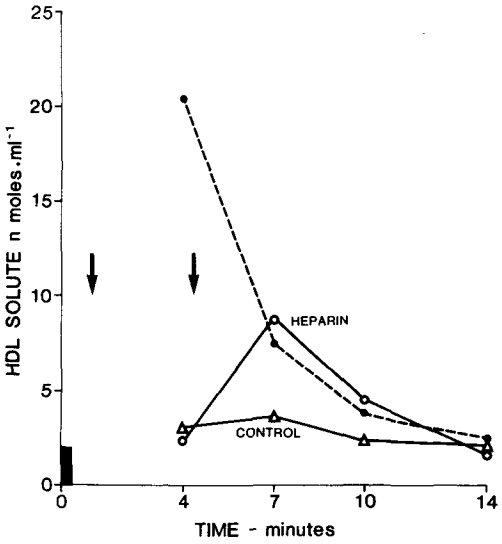

FIG. 6. Transfer of [^{14}C]hydrocarbon to HDL from chylomicrons during heparin stimulation of chylomicron triacylglycerol hydrolysis. Chylomicrons containing [^{14}C]hexadecane and [^{3}H]triacylglycerol, 2.1 μmol and 45 μmol/kg body wt respectively, were injected (dense bar) in 3 animals, wt 280 g. In 2, heparin was subsequently injected intravenously (arrows). △——△, control; ●——●, heparin at 1 min; ○——○, heparin at 4½ min. Percentage of plasma hydrocarbon in HDL at 4 min, 7 min, 10 min and 14 min were, respectively, control 7%, 10%, 8% and 9%, 1 min heparin 65%, 27%, 17% and 17% and 4 min heparin 7%, 25%, 33% and 22%.

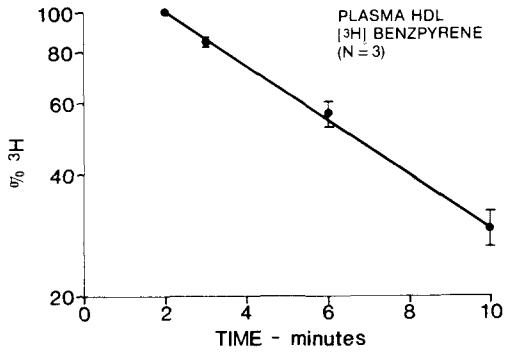

FIG. 7. Plasma HDL was serially sampled after pulse injection of HDL containing [^{3}H]benzopyrene (Fraction III column eluate obtained from chylomicron [^{3}H]benzopyrene injected animals [n = 3]). Chylomicron benzopyrene load injected was 264 ± 160 pg/kg body weight. Details are in text. Means ± SEM.

pulse and after plasma sampling. Three chylomicron batches (1 octadecane and 2 hexadecane) were injected in 3 experimental groups, each containing 1 saline-injected control and 1 or 2 heparin-injected animals (n = 5). Heparin injection after adequate chylomicron-plasma mixing (4 min) induced a rapid decrease in plasma [^{3}H]triacylglycerol in the subsequent 3 min (88 ± 2% [n = 3] vs 32 ± 3% in controls). Plasma [^{14}C]alkane radioactivity in HDL at 4 min, 7 min and 10 min was 100%, 104 ± 1.6% and 71 ± 8%, respectively, in controls, but after heparin it increased by 177 ± 55% (n = 12, $P < 0.01$) above control values at the corresponding sample times at 4 min, 7 min or 10 min. HDL [^{14}C]alkane formed 7-24% of total plasma lipoprotein [^{14}C]alkane in controls and increased to 16-65% in post-heparin plasmas. The results for one typical experimental group are shown in Figure 6. Post-heparin HDL prepared by ultracentrifugation gave similar results to precipitated HDL in 7 min sera (n = 4), respectively 93 ± 6% and 107 ± 6% of combined mean HDL [^{14}C]alkane. Following the peak of HDL [^{14}C]alkane after heparin, the clearance rate of HDL [^{14}C]alkane from plasma ($k = 2.7 \pm 0.5 \times 10^{-3}$/sec, n = 5) exceeded control clearance rates for the same 3 min interval ($k = 0.8 \pm 0.7 \times 10^{-3}$/sec, n = 5, $P < 0.05$). This indicated that high [^{14}C]alkane in HDL after heparin was caused by increased influx and not by inhibited effux. These results are interpreted as evidence for the importance of lipoprotein lipase in mediating chylomicron-HDL solute transfer.

HDL are heterogeneous in composition and metabolic derivation. Comparison of all 4 HDL analytical methods gave similar quantitative results for HDL hydrocarbon and the major plasma acceptor of hydrocarbons was characterized as HDL by precipitation, agarose chromatography, density flotation and electrophoresis.

The plasma kinetics and tissue disposition of these HDL hydrocarbons were examined only for [^{3}H]benzopyrene because alkanes are potentially exchangeable with endogenous pools and DDT radioactivity in HDL was insufficient. Lymph chylomicrons with [^{3}H]-benzopyrene were obtained from 3 animals and after injection (n = 3) HDL labeled with [^{3}H]-benzopyrene were obtained from Peak III of agarose chromatograms, dialyzed and reinjected. HDL [^{3}H]benzopyrene in pulse injections of 1.0 ± 0.1 mg HDL cholesterol were cleared rapidly from plasma with a single exponential disappearance ($t_{½}$ = 4.6 ± 0.5 min, n = 3). The results are shown in Figure 7. All plasma samples were examined for [^{3}H]benzopyrene transfer to lower density lipoproteins but significant ^{3}H was not detected in these. Livers excised 10 min after HDL injection in 2 experiments contained 39% and 46% of the ^{3}H cleared from plasma. Liver ^{3}H per g wet tissue

TABLE 5

Chylomicron [^{14}C] DDT Transfer to Proteins in Vitro

Additions to chylomicron incubate					Percentage of ^{14}C DDT at gradient origin
	Nil	Albumin		Serum	
Protein g/dl	0	5.0		4.8	
	+				0.4
		+			0.7
				+	20.0
	Nil	Alb	Alb	HDL	VHDL	
Protein g/dl	0	3.75	0.15	0.15	2.0	
	+					0.5
		+				5.9
			+			0.7
				+		10.6
					+	5.8
		+		+		14.3
				+	+	13.9

Chylomicrons were incubated at 37 C for 10 min with proteins at mean 8 mM chylomicron triacylglycerol and molar DDT concentration in triacylglycerol of 0.001% in 2 experiments. Incubate chylomicrons were separated by flotation from denser proteins and HDL at sucrose gradient origin. Chylomicrons were from different animals for each experimental group. Results are duplicate assay means.

was 15-fold greater than that in the control tissue, psoas muscle. These results suggest that [^{3}H]benzopyrene in plasma HDL was cleared directly to tissues without major transfer to other lipoproteins and that liver was a major acceptor of HDL [^{3}H]benzopyrene.

To determine if solute transfers from chylomicrons observed in vivo existed in vitro, chylomicrons labeled with [^{14}C]DDT or [^{14}C]-octadecane were incubated with serum, bovine serum albumin or with serum lipoprotein fractions. Subsequent flotation in sucrose separated chylomicrons from HDL and denser proteins, which remained at the origin. [^{14}C]-DDT transferred readily and the results are shown in Table 5. In experiment 1, bovine serum albumin bound 1.71 μg DDT per g, but serum total proteins bound 5.0 μg DDT per g and 20% of chylomicron DDT. Other chylomicrons were incubated with albumin or lipoproteins and again separated from these proteins by flotation in sucrose gradients (experiment 2, Table 5). HDL bound most [^{14}C]DDT at the origin even at low HDL concentrations. The addition of HDL to other fractions increased the percentage of DDT at origin for saline, albumin and VHDL by 10.1%, 8.4% and 8.1%, respectively. The μg amounts of DDT bound per g protein were 0.05 for albumin, 2.23 for HDL and 0.09 for VHDL. These results are consistent with rapid diffusion of DDT from chylomicrons and a high affinity of DDT for HDL, 45 times greater than that for albumin at the physiologic concentrations of proteins used in these experiments. Octadecane, in contrast, did not transfer from chylomicrons to HDL either in preliminary experiments analogous to the DDT incubation or where prolonged incubation and Mg^{++} precipitation were used (Table 6). These results indicate that in vitro efflux of chylomicron DDT is rapid and quantitatively important but no evidence of octadecane efflux in vitro was found. The in vitro results, in the absence of lipoprotein lipase activity, are consistent with the in vivo findings of rapid DDT clearance from chylomicron triacylglycerol, which is partly independent of triacylglycerol clearance, and with the apparent complete dependence of octadecane clearance on triacylglycerol clearance.

DISCUSSION

The present study describes the major characteristics of a lipoprotein transport system that transports hydrocarbons of low polarity from the intestinal lumen into lymph lipoproteins and then via plasma chylomicrons to plasma HDL. Four compounds with a range of polarity from benzopyrene to octadecane and with 3 widely differing molecular structures, were absorbed from gut into intestinal lymph

TABLE 6

Chylomicron [^{14}C] Octadecane Transfer to HDL in Vitro

	Net percentage of incubate [^{14}C] octadecane in HDL					
Incubation, temperature 0 C	<0.5	0.5	<0.5	0.8	<0.5	0.5
37 C	0.8	0.7	<0.5	0.6	<0.5	0.8
Incubation, minute	0	5	10	20	30	60

Chylomicrons containing [^{3}H] triacylglycerol and [^{14}C] octadecane were incubated at 1.2 mM triacylglycerol and 4.1 μM octadecane in 90% rat serum. HDL was measured by mg^{++} precipitation and supernatant [^{14}C] chylomicron contamination was corrected by ^{3}H assay (<2.1% per incubate). Results are means of duplicates.

chylomicrons. The potential quantitative importance of this pathway was high since, after high fed loads, octadecane and DDT were recovered at 1-3% concentration in lymph chylomicron triacylglycerol. The quantitative association of all 4 hydrocarbons with triacylglycerol supports the suggestion that core lipid provides an oil phase for nonspecific transport of hydrocarbons of low polarity (8). Efficiency of intestinal absorption and partition of solutes between portal and lymphatic systems were not examined in this study. Little information is available on the absorption and subsequent transport of quantitatively minor lymph lipids. Aliphatic hydrocarbons with carbon numbers from C14 to C32 fed intragastrically to rats are absorbed with an efficiency that correlates inversely with their carbon number (25). Gut absorption and recovery in lymph have been reported for [^{14}C]cetyl alcohol (6) with 50% of the former absorbed and 70% of this was recovered in intestinal lymph. High efficiency of intestinal absorption and transport into intestinal lymph has been reported with [^{14}C]-DDT with 60% recovered from lymph within 12 hr of gastric feeding (8).

The subsequent transport in plasma of xenobiotic absorbed hydrocarbons in intestinal lipoproteins had been studied only with DDT (8). More information is available for the physiological lipids transported in minor quantity in intestinal lipoproteins, i.e., Vitamins A, D and E. Vitamin A as retinal or retinol, is absorbed efficiently and transported esterified in lymph in chylomicrons (1). Vitamin A in chylomicrons injected intravenously in rats, is taken up rapidly by liver (1) and is found only in chylomicrons or chylomicron remnant particles prior to hepatic uptake (26). Vitamin D_2 and D_3, following intestinal absorption, are recovered in lymph, predominantly in chylomicrons, but plasma chylomicron transport has not been studied. Tocopherols are associated with chylomicrons in human lymph (27) and α- and γ-tocopherol are efficiently absorbed, entering the VLDL-chylomicron fraction of rat lymph. Chylomicron [^{3}H]tocopherol injected intravenously or incubated in serum is recovered in low and high density plasma lipoproteins, results interpreted as redistribution in proportion to lipoprotein lipid mass (3). The nature of transport of hydrocarbons from lipoproteins is complex. Results with hydrocarbons introduced into lipoproteins in vitro indicate subsequent passive diffusion of benzopyrene and its hydroxylated metabolites among major human serum lipoproteins (29) and similar diffusion of chlorinated hydrocarbons (30). Simple passive diffusion of hydrocarbons introduced biologically in lipoproteins has been reported only for chylomicron DDT (8). In the present study, 2 methods of hydrocarbon clearance from chylomicrons were determined: (a) simple passive diffusion from intact chylomicrons, independent of hydrocarbon concentration in triacylglycerol and partly independent of triacylglycerol hydrolysis and (b) clearance dependent on triacylglycerol hydrolysis but independent of hydrocarbon concentration. The latter is consistent with passive diffusion facilitated by lipoprotein-lipase and its induced molecular changes in chylomicrons. These are binding to the enzyme at endothelial cell surfaces (24), subsequent release of redundant polar surface components (31), transfer of apoproteins and phospholipids to HDL with transient formation of particles in the LDL and HDL particle diameter range (22) and the return of C apoproteins to HDL (32). In addition to these changes, which provide potential hydrocarbon transport pathways, hydrocarbons may be retained in chylomicron remnant particles for subsequent clearance. Although no relative retention of hydrocarbons occurred in agarose Peak I, which contains remnant particles, this fraction is heterogeneous and one other lipid, [^{14}C] cholesterol, is cleared very rapidly from plasma enriched in remnant particles (28). A major role for remnants in hydrocarbon transport cannot be

excluded.

[^{3}H]Benzopyrene in vivo did not transfer rapidly to other lipoproteins either from chylomicrons or from injected HDL. This differs from a report in which chylomicrons, labeled with [^{3}H]benzo(α)pyrene by incubation, were injected in rats and [^{3}H]benzopyrene was recovered predominantly in VLDL and the ultracentrifuged residual protein fraction ($d > 1.21$) with highest specific radioactivity in lung (33). These differences may reflect incubation damage to chylomicrons and ultracentrifugation shear damage to VLDL and chylomicrons that generates lipoprotein material in the residual protein fraction (12).

The liver was a quantitatively important site of rapid hydrocarbon clearance for all 4 hydrocarbons of chylomicrons and for HDL benzopyrene. Chylomicron octadecane was not cleared more rapidly than triacylglycerol in intact animals and its clearance rate was markedly depressed in the hepatectomized rats in the face of continuing triacylglycerol clearance. These results suggest that the liver clears octadecane from lipoprotein products of peripheral triacylglycerol degradation of chylomicrons and not from intact chylomicrons.

That the transfer of hydrocarbons from chylomicrons to HDL was correlated with triacylglycerol hydrolysis was suggested by the absence of alkane transfer from unhydrolyzed chylomicrons in vitro and by the simultaneous increase in hydrolysis and alkane transfer to HDL induced in vivo by heparin. Lipoprotein lipase inhibition in vivo was not studied because the inhibitors, galactosamine, cycloheximide and protamine, also profoundly alter HDL metabolism. We did not establish whether hydrocarbon transfer, with or without heparin, was to preexisting HDL or to HDL newly generated by transfer of other chylomicron components to the HDL fraction during lipoprotein lipase action (31). The molecular characteristics of the hydrocarbon transporting HDL were, by 4 methods, those of classical HDL but plasma HDL is highly heterogeneous (34) as is its plasma production by direct secretion from liver (35) and intestines (36) and by metabolic degradation of chylomicrons (33).

HDL selectively concentrated hydrocarbons from their chylomicron triacylglycerol phase both during passive transfer of DDT from unhydrolyzed chylomicrons in vitro and with other solutes in vivo. The report of long-chain alkanes, including octadecane, in human HDL (37) and the demonstration of octadecane in normal rat HDL in this study, in the absence of reports of octadecane synthesis in mammalian systems, support the concept of HDL as an acceptor of nonpolar solutes from intestinal lipoproteins.

HDL have an established role as a reservoir of apoproteins that transfer to triacylglycerol-rich lipoproteins entering plasma (32) stimulating peripheral hydrolysis and blocking recognition of triacylglycerol-rich lipoproteins by hepatic receptors (38). HDL reduce binding, interiorization and degradation of LDL in a variety of peripheral cells, a characteristic of HDL that may reside exclusively in the arginine-rich protein subfraction of HDL (39). The function of HDL in transporting lipid is controversial. HDL may have an important quantitative role in removal of peripheral cell cholesterol to plasma by uptake and interiorization in HDL after esterification by LCAT (40). Direct net chemical clearance of HDL cholesterol has not been demonstrated during splanchnic passage (41) but kinetic analysis indicates that free cholesterol in HDL is a preferred substrate for biliary cholesterol secretion in man (42). HDL cholesterol transfers to VLDL in the presence of cholesterol transfer protein (43). This may provide an indirect route for peripheral cell cholesterol transfer via HDL to liver. The present results indicate a second lipid transport role of HDL for nonpolar hydrocarbons absorbed from gut and suggest that HDL may have a specific function among rat plasma proteins of accepting and concentrating solute hydrocarbons from triacylglycerol-rich lipoproteins. The transport of a known cellular toxin (DDT) and a carcinogen (benzopyrene) suggest potential importance for this system in cell pathology.

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REFERENCES

1. Smith, J.E., Muto, Y., Milch, P.O., and Goodman, D.S. (1973) J. Biol. Chem. 248, 1544-1549.
2. Schachter, D., Finkelstein, J.D., and Kowarski, S. (1964) J. Clin. Invest. 43, 787-796.
3. Peake, I.R., Windmueller, H.G., and Bieri, J.G. (1972) Biochim. Biophys. Acta 260, 679-688.
4. McCormick, E.C., Cornwell, D.G., and Brown, J.B. (1960) J. Lipid Res. 1, 221-228.
5. Savary, P., and Constantin, M.J. (1967) Biochim. Biophys. Acta 137, 264-276.
6. Baxter, J.H., Steinberg, D., Mize, C.E., and Avigan, J. (1967) Biochim. Biophys. Acta 137, 277-290.

7. Grubbs, C.J., and Moon, R.C. (1973) Cancer Res. 33, 1785-1789.
8. Pocock, D.M., and Vost, A. (1974) Lipids 9, 374-381.
9. Zilversmit, D.B. (1968) J. Lipid Res. 9, 180-186.
10. Rudel, L.L., Lee, J.A., Morris, M.D., and Felts, J.M. (1974) Biochem. J. 139, 89-95.
11. Havel, R.J., Eder, H.A., and Bragdon, J.H. (1955) J. Clin. Invest. 34, 1345-1353.
12. Herbert, P.N., Forte, T., Shulman, R., Lapiana, M., Gond, E., Levy, R., Fredrickson, D.S., and Nichols, A.V. (1975) Prep. Biochem. 5, 93-129.
13. Imaizumi, K., Fainaru, M., and Havel, R.J. (1978) J. Lipid Res. 19, 712-722.
14. Lees, R.S., and Hatch, F.T. (1963) J. Lab. Clin. Med. 61, 518-528.
15. Burstein, M., Scholnick, H.R., and Morfin, R. (1970) J. Lipid Res. 11, 583-595.
16. Wieland, O. (1957) Biochem. Z. 329, 313-317.
17. Allain, C.C., Poon, L.S., Chan, C.S.G., Richmond, W., and Fu, P.C. (1974) Clin. Chem. 20, 470-475.
18. Gustafson, A., Alaupovic, P., and Furman, R.H. (1965) Biochem. 4, 596-605.
19. Flanagan, V.P., and Ferretti, A. (1973) J. Lipid Res. 14, 306-311.
20. Pocock, D.M.E., Rafal, S., and Vost, A. (1972) J. Chromatographic Science 10, 72-76.
21. Vost, A. (1972) J. Lipid Res. 13, 695-704.
22. Sabesin, S.M., Kuiken, L.B., and Ragland, J.B. (1975) Science 190, 1302-1304.
23. Tall, A.R., Green, P.H.R., Glickman, R.M., and Riley, J.W. (1979) J. Clin. Invest. 64, 977-989.
24. Blanchette-Mackie, E.J., and Scow, R.O. (1971) J. Cell Biol. 51, 1-25.
25. Albro, P.W., and Fishbein, L. (1970) Biochim. Biophys. Acta 219, 437-446.
26. Ross, A.C., and Zilversmit, D.B. (1977) J. Lipid Res. 18, 169-181.
27. Bieri, J.G., and Farrell, P.M. (1976) Vitamins and Hormones 34, 31-75.
28. Redgrave, T.G. (1970) J. Clin. Invest. 49, 465-471.
29. Shu, H.P., and Nichols, A.V. (1981) Biochim. Biophys. Acta 665, 376-384.
30. Maliwal, B.P., and Guthrie, F.E. (1982) J. Lipid Res. 23, 474-479.
31. Redgrave, T.G., and Small, D.M. (1979) J. Clin. Invest. 64, 162-171.
32. Havel, R.J., Kane, J.P., and Kashyap, M.L. (1973) J. Clin. Invest. 52, 32-38.
33. Vauhkonen, M., Kuusi, T., and Kinnunen, P.K.J. (1980) Cancer Letters 11, 113-119.
34. Suenram, A., McConathy, W.J., and Alaupovic, P. (1979) Lipids 14, 505-510.
35. Hamilton, R.L., Williams, M.C., Fielding, C.J., and Havel, R.J. (1976) J. Clin. Invest. 58, 667-680.
36. Green, P.H.R., Tall, A.R., and Glickman, R.M. (1978) J. Clin. Invest. 61, 528-534.
37. Skipski, V.P., Barclay, M., Barclay, R.K., Fetzer, V.A., Good, J.J., and Archibald, F.M. (1967) Biochem. J. 104, 340-352.
38. Windler, E., Chao, Y., and Havel, R.J. (1980) J. Biol. Chem. 255, 5475-5480.
39. Mahley, R.W., and Innerarity, T.L. (1977) J. Biol. Chem. 252, 3980-3986.
40. Glomset, J.A. (1968) J. Lipid Res. 9, 155-167.
41. Sniderman, A., Thomas, D., Marpole, D., and Teng, B. (1978) J. Clin. Invest. 61, 867-873.
42. Schwartz, C.C., Halloran, L.G., Vlahcevic, Z.R., Gregory, D.H., and Swell, L. (1978) Science (Washington, DC) 200, 62-64.
43. Barter, P.J., and Lally, J.I. (1979) Biochim Biophys. Acta 572, 510-518.

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Differential Response of Lipid Metabolism and Membrane Physical State by an Actively and Passively Overwintering Planktonic Crustacean

T. FARKAS,[a]* GY NEMECZ[a] and I. CSENGERI,[b] [a]*Institute of Biochemistry, Biological Research Center, Hungarian Academy of Sciences, H-6701 Szeged, Hungary and* [b]*Fish Culture Research Institute, H-5541 Szarvas, Hungary*

ABSTRACT

Phospholipid (PL) composition, fatty acid (FA) composition of total and major individual PL as well as the physical state of isolated PL were investigated in relation to temperature exposure by freshwater planktonic crustaceans, *Cyclops vicinus* and *Daphnia magna*. *C. vicinus*, adapted to warm water, accumulated appreciable amounts of docosahexaenoic acid in its PL within 3 days when the temperature was decreased from 20 C to 10 C. Docosahexaenoic acid was preferentially esterified to phosphatidylethanolamine (PE). Docosapolyenoic FA were absent in PL of *D. magna* and this species did not increase polyenoic acid level under identical temperature treatment. The level of PE was elevated, however, in both species in response to decreased environmental temperature. Two characteristic breaks were observed in ln S vs 1/T plots of 5-doxyl stearic acid spin probe. These were at 19 C and 13 C for *C. vicinus* and at 20 C and 7 C for *D. magna*. *C. vicinus* shifted both the upper and lower phase-separation temperatures of its PL to lower temperatures when exposed to cold. Differences between the onset and completion of phase-separation temperatures equalled that in environmental temperature (10 C). The phase-separation temperatures of *D. magna* were unchanged under identical experimental conditions. Results are interpreted as a complete temperature adaptation of membrane transitional state by *C. vicinus* but not by *D. magna*. Researchers have postulated that one of the reasons the latter species cannot overwinter in an active form as does *C. vicinus*, but instead overwinters as resting eggs, involves its failure to adapt membrane PL composition and physical state to temperature.

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INTRODUCTION

Investigations made on microbial systems (1-4), plants (5-7) and some poikilothermic animals (8,9) showed an adaptation of membrane lipid composition and physical state to acclimation temperature. Some data show that maximum and minimum growth temperatures of some microorganisms depend on the cell's ability to maintain proper physical characteristics of their membrane structures (10,11). On the basis of these data, the assumption can be made that seasonal occurrence or even geographical distribution of different species may partially be determined by whether they can manipulate their membranes in order to survive at a given temperature. We found earlier, by investigating changes in phospholipid (PL) fatty acid (FA) composition, that those planktonic crustaceans unable to increase long-chain polyunsaturated FA in their PL did not spend the winter in active form whereas those active in winter regulated the level of these FA according to the temperature (12,13). Thus, crustaceans belonging to the latter group can be expected to actively adapt the physical state of their membranes to changing temperatures. PL polar head group composition, FA composition of phosphatidylcholines (PC) and phosphatidylethanolamines (PE) and order-disorder transitions of PL vesicles obtained from *Cyclops vicinus* and *Daphnia magna* adapted to warm temperatures, then shifted to cooler temperatures, were investigated in this study as a test of this hypothesis. The former species is abundant in natural waters at a rather wide temperature range (5-20 C) whereas the latter exists only above 10-12 C.

*To whom correspondence should be addressed.

MATERIALS AND METHODS

Animals

C. vicinus and *D. magna* were collected from different fish ponds on May 10 and May 7, 1982, respectively. Water temperature in these ponds varies between 2-3 C and 25-30 C in winter and summer. The actual temperature at the time of collecting was 18 C for *C. vicinus* and 20 C for *D. magna*. The population of *C. vicinus* was declining when collected. The populations were nearly homogenous (>95%). The animals were brought to the laboratory alive and placed in 30 l aquaria, containing filtered lake water. Filtering removed any kind of suspended material, including green algae

and diatoms. One group (25 g fresh weight) of animals was killed immediately by adding chloroform/methanol (2:1, v/v). The other animals in the aquaria were placed in a cold room (10 C), and were allowed to cool down over a period of 20 hr. After 72 hr in the cold room, they were removed from the tanks by filtration and treated as above.

Extraction and Separation of Lipids

The Folch extraction technique (14) was used to obtain total lipids. Total lipids were separated into neutral and polar lipid fractions by silicic acid column chromatography. Polar lipids, eluted with methanol, were stored at −25 C in chloroform containing 0.01% butylated hydroxytoluene as antioxidant. In some other cases, one-dimensional thin layer chromatography (TLC), using petroleum ether/ethyl ether/acetic acid (85:15:1, v/v/v) as solvent, was used to separate lipid classes. PL class composition was determined by two-dimensional TLC (15). One-dimensional TLC with a solvent system of chloroform/methanol/water (65:25:4, v/v/v) was used to obtain pure PC and PE. Visualization was accomplished by spraying the plates with 0.2% 8-aminonaphthalene sulfonic acid in 50% methanol and inspecting the plates under ultraviolet (UV) light. PL standards were purchased from Serdary Research Laboratories (London, Ontario, Canada).

FA composition of total as well as of purified individual PL were determined by gas liquid chromatography (GLC) following transesterification in the presence of 5% HCl in absolute methanol at 80 C. A JEOL 20K instrument, equipped with dual flame ionization detector (FID) was used. The separations took place on 15% EGSS-X (Applied Science Lab., Deerfield, IL) coated onto 100-120 mesh Supelcoport (Supelco, Bellefonte, PA). Temperature was programmed with a rate of 2 C per min from 140 C to 195 C. Identification was made using reference standards (Nu-Chek Prep, Elysian, MN; Serdary Research Lab.) and plotting the log of relative retention times vs carbon atoms, obtained in isothermal runs. Quantitation was done by an electronic integrator (Packard 603). Each sample was run in triplicate.

Electron Spin Resonance Spectroscopy

PL (0.4-0.5 $\times$ 10^{-6} mol) was mixed with 15-20 nmol 5-doxyl stearate [5-(N-oxy-4',4'-dimethyloxazolidino)-stearic acid] at room temperature for 5 min. The samples were poured into microflat cell (Scanlon) and temperature programmed from 0 C to 25 C. Spectra were recorded with a JES-PE-1X (JEOL) Electron Spin Resonance Spectrometer. Order parameter (S) was calculated by the method of Seelig (16).

RESULTS

Effect of Cold Exposure on PL FA Composition

In order to ascertain whether the investigated species behave identically as found previously (12), the animals adapted to warm temperatures were exposed to 10 C for 72 hr and the PL FA compositions were compared with the initial states. The data of Table 1 support earlier observations regarding the differences in FA composition of copepods and cladocerans (12), i.e., high amounts of docosapolyenoic FA in the former and the absence in the latter species. The response to decreased temperature by the 2 species was similar only in one respect: both left the proportion of palmitic acid unchanged. The levels of C18 saturated and unsaturated fatty acids were reduced in *C. vicinus*. This animal did not change the level of 20:5ω3, the second most abundant long-chain polyunsaturated FA but increased 22:6ω3 by ca. 13%. Total number of double bonds also increased by ca. 15%. *D. magna* exposed to cold did not have elevated levels of polyunsaturated FA in their PL and, in contrast to *C. vicinus*, did not have reduced C18 fatty acids (including stearic acid) levels. Consequently, the total number of double bonds also remained unchanged.

PE are the major acceptors of 22:6 in *C. vicinus* (Table 2). Only PC and PE were analyzed in this study. The fact, however, that the level of docosahexaenoic acid in animals exposed to cold was less than in the total PL fraction indicates that this acid may have been incorporated in appreciable amounts into some other PL. Palmitic acid level increased in PC on exposure to cold and was almost identical to that of docosahexaenoic acid in both control and *C. vicinus* exposed to cold. The same is not true for PE. Detailed investigations on molecular-species composition are in progress to reveal how exposure to cold affected the pairing of saturated and unsaturated FA. In *D. magna* exposed to cold, almost no changes occurred in PC and PE FA compositions except for an elevated level of palmitic acid.

Phospholipid Compositions in Animals Shifted to Cooler Temperatures

PC was roughly 50% of the total PL in *D. magna* adapted to warm temperatures. PE was the second most abundant PL in this

TABLE 1

Effect of Shift of Temperature from 20 C to 10 C on Fatty Acid Composition of Total Phospholipids in *Cyclops vicinus* and *Daphnia magna*

Species	*C. vicinus*		*D. magna*	
Temperature C	20	10	20	10
Fatty acids			mol (%)	
14:0	1.7	1.0	0.6	0.8
14:1	0.4	0.6	0.3	0.7
15:0	0.5	0.6	–	–
16:0	19.8 ± 0.1	20.0 ± 0.1	20.3 ± 0.2	21.3 ± 0.1
16:1	2.3	2.6	4.2	4.4
16:2	2.5	3.0	3.6	2.0
18:0	6.1 ± 0.1	1.1 ± 0.1	5.2 ± 0.1	5.2 ± 0.1
18:1	3.6	1.9	20.9 ± 0.1	20.0 ± 0.1
18:2	2.2	0.1	4.1	4.3
18:3	7.6	5.4	13.4 ± 0.1	13.6 ± 0.1
20:1	0.6	0.3	2.4	2.1
20:2	0.3	0.4	0.1	0.1
20:3	tr	tr	0.3	tr
20:4	1.7	1.2	6.9	6.7
20:5	14.9 ± 5	14.2 ± 0.6	17.7 ± 0.1	18.4 ± 0.1
22:4	0.5	tr	–	–
22:5	2.3	2.5	–	–
22:5	3.1	3.4	–	–
22:6	30.0 ± 0.9	41.7 ± 1.0	–	–
Σ Double bonds	322	383	200	189
Saturated / unsaturated	0.4	0.3	0.3	0.4

The values are the averages ± SE of 3 determinations from a pooled sample.

species. On the contrary, *C. vicinus* adapted to warm temperatures was richer in PE than in PC. The ratios of PC to PE were 1.6 and 0.8 in *D. magna* and *C. vicinus* adapted to warm temperature. The response to decreased temperature was similar: PC decreased with a simultaneous increase in PE levels in both species. The ratio of PC to PE had decreased to 1.1 and 0.5 in the respective species. Lysophosphatidylcholine was absent but a ninhydrin positive spot, showing identical chromatographic behavior to lysophosphatidylethanolamine when cochromatographed, showed a tendency to decrease when exposed to cold. The same also was true for sphingomyelin, whereas phosphatidylserine tended to accumulate.

Ordering State of Isolated PL in Normal Animals and Animals Shifted to Cooler Temperatures

Two characteristic breaks are found on ln S vs 1/T plots occurring at 19 C and 13 C and 20 C and 7 C in *C. vicinus* (Fig. 1) and *D. magna* (Fig. 2), respectively, adapted to warm temperatures. The break at the higher temperature is regarded as the onset, whereas that at the lower temperature is regarded as the completion of phase separation (17). Although the FA composition of total PL as well as the polar head composition differ markedly, the S values are almost identical in both crustaceans. Evidently, both of these parameters made contributions in order to assure identical physicochemical characteristics to the membrane PL in animals grown at 20 C.

Shift of the temperature from 20 C to 10 C resulted in an increase of order parameters in *C. vicinus*, which would indicate some increase in molecular packing. However, both the onset and completion of phase separation temperatures were shifted to 10 C and 3 C, respectively (Fig. 1). The magnitude of shifting to cooler phase separation temperatures exactly corresponded with that of the environmental water temperature. In contrast, the same treatment had no influence on the physical state of PL in *D. magna* (Fig. 2). Neither the S values nor the break-point temperatures change when exposed to cold.

DISCUSSION

The present data confirm earlier observations on FA composition of freshwater cladocerans and copepods (12) and on the differential re-

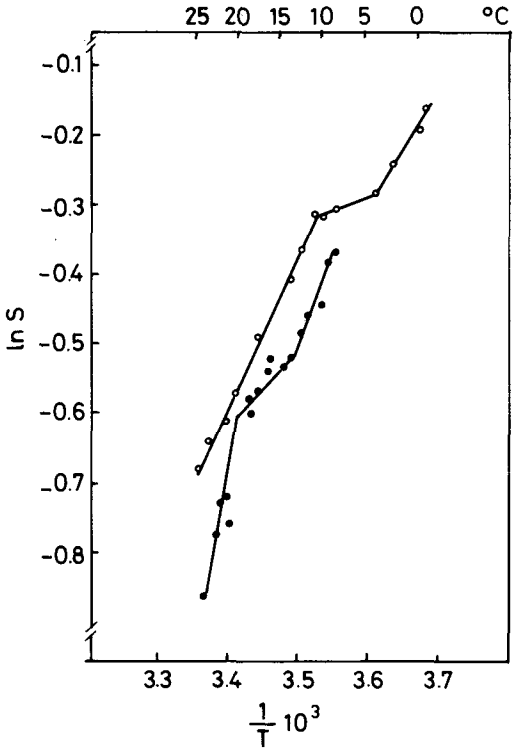

FIG. 1. Temperature dependency of the order parameter, S, of 5-doxyl stearic acid in isolated phospholipids on *C. vicinus* adapted to warm temperatures placed in cooler temperatures; • = warm adapted, ○ = exposure to 10 C for 3 days. The standard error of order parameter, S, was 2%.

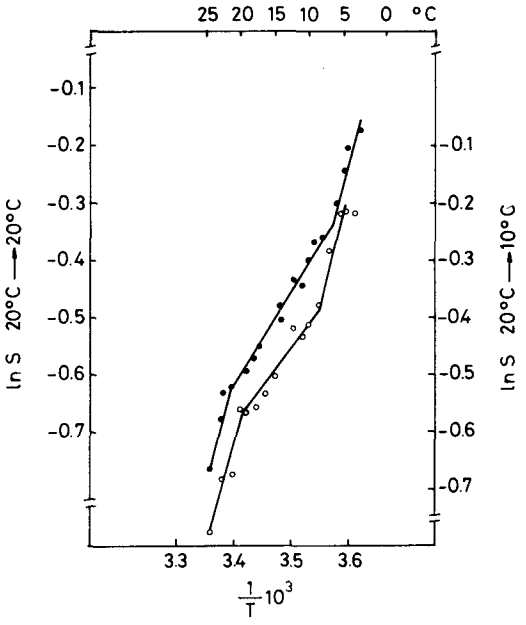

FIG. 2. Temperature dependency of the order parameter, S, of 5-doxyl stearic acid in isolated phospholipids of *D. magna* adapted to warm temperatures placed in cooler temperatures; • = warm adapted, ○ = exposure to 10 c for 3 days. Note that the ordinate for the plot of animals placed in cooler temperatures is shifted one unit downwards to avoid overlapping.

sponse of lipid metabolism of various crustaceans to changes in environmental temperature (13). A basic difference between *C. vicinus,* a representative species active in winter and summer, and *D. magna,* representing those active only in the summer, is that only the former could increase the level of long-chain polyunsaturated FA in PL when exposed to cold. Accumulation of long-chain polyunsaturated FA in *C. vicinus* exposed to cold is not likely to be the result of a selective retention from possible starvation during the experiment. Previous studies using labeled precursors showed an immediate rise in formation of these FA in cyclops shifted to lower temperatures (13). Biosynthesis of long-chain polyunsaturated FA proceeds via chain elongation and desaturation of the corresponding precursors (18). As no food was available during the experiment, these precursors must have been liberated from some complex lipids. Thus, a rapid temperature-induced deacylation followed by chain elongation, desaturation and reacylation can be postulated to take place on exposure to cold. This speculation is supported by observations of Chapelle et al. (19) showing that the temperature controls the phosphatidylcholine ⇌ lysophosphatidylcholine deacylation-reacylation reaction in the decapod crustacean, *Carcinus meanas.* Dickens and Thompson (20) suggest this process plays an important role in thermal adaptation of *Tetrahymena.* Although we do not have direct evidence to prove this point, the reduction of lysophosphatidylethanolamine in *C. vicinus* and *D. magna* exposed to cold (Table 3) fits well in this picture. As *D. magna* did not increase the production of long-chain polyunsaturated FA with exposure to cold it must recycle the FA already present; hence, its PL FA composition remains unchanged.

Besides PL FA compositions, the PL polar head group composition is also different in the 2 crustaceans investigated (Table 3). However, despite significant differences in PL class and FA composition of crustaceans adapted to warm temperatures, the ordering states of isolated PL were identical (Figs. 1 and 2). Also, the onset of phase separations were at identical temperatures and exactly at the temperature of growth. Thus, as in some unicellular systems

TABLE 2

Fatty Acid Composition of Phosphatidylethanolamines and Phosphatidylcholines in *Cyclops vicinus* and *Daphnia magna* Before and After the Shift of Temperature from 20 C to 10 C

	Phosphatidylcholine				Phosphatidylethanolamine			
Species	*C. vicinus*		*D. magna*		*C. vicinus*		*D. magna*	
Temperature C	20	10	20	10	20	10	20	10
Fatty acids				mol (%)				
14:0	4.1	2.9	1.3	2.5	0.9	2.5	1.2	3.9
14:1	0.7	0.3	0.1	0.1	0.2	0.3	0.1	0.1
15:0	0.7	0.3	2.3	1.2	0.1	0.1	0.3	1.3
16:0	23.2±0.6	28.3±0.8	11.3±0.4	23.5±0.7	22.2±0.8	19.9±0.7	15.1±0.3	16.7±0.6
16:1	3.1	4.1	9.6	8.6	3.0	2.9	9.1	9.4
18:0	0.9	2.3	7.8	4.8	11.0±0.1	11.1±0.2	4.1	4.0
18:1	1.7	8.8	18.1±0.6	15.6±0.2	3.9	3.9	13.9±0.4	15.0±0.4
18:2	2.7	1.7	8.2	6.2	1.8	1.0	13.8±0.4	15.5
18:3	11.1±0.2	7.0±0.3	14.6±0.2	12.6±0.4	7.4±0.1	4.1±0.1	15.1±0.5	11.8±0.3
20:1	0.5	0.4	3.1	2.4	0.8	0.2	3.1	2.3
20:4	2.6	1.8	2.6	2.7	2.7	1.9	7.1	3.1
20:5	23.7±0.7	15.1±0.5	19.3±0.8	19.4±0.7	10.8±0.2	9.3±0.4	16.3±0.8	16.7±0.7
22:4	1.0	0.7	–	–	–	–	–	–
22:5	–	–	–	–	2.4	2.7	–	–
22:5	1.3	1.6	–	–	1.9	2.7	–	–
22:6	22.6±0.4	25.6±0.7	–	–	30.6±0.9	37.4±1.0	–	–

For legend see Table 1.

TABLE 3

Phospholipid Composition of *Cyclops vicinus* and *Daphnia magna* Before and After Shift of Temperature from 20 C to 10 C

Species	*C. Vicinus*		*D. magna*	
Temperature C	20	10	20	10
Phosphatidylinositol	6.3 ± 0.3	5.7 ± 0.1	5.2 ± 0.2	8.7 ± 0.4
Phosphatidylserin	2.7 ± 0.2	8.2 ± 0.9	2.5 ± 0.5	3.6 ± 0.7
Sphyngomyelin	3.1 ± 0.5	1.2 ± 0.1	5.2 ± 0.5	3.9 ± 0.7
Lysophosphatidyl-ethanolamine	5.4 ± 0.5	5.2 ± 0.2	2.5 ± 0.1	—
Phosphatidylcholine	37.5 ± 1.6	27.7 ± 0.8	52.6 ± 1.0	43.4 ± 2.0
Phosphatidylethanolamine	44.9 ± 2.6	54.8 ± 0.6	31.9 ± 1.4	40.6 ± 2.1

For legend see Table 1.

(1,2,21,22), a very close correlation is found between growth temperature and membrane physical state in these crustaceans. As revealed by Tables 2 and 3, *C. vicinus* and *D. magna* adapted to warm temperatures achieved identical fluidity parameters following different routes. PC form less packed membranes than PE of the same FA composition (23) but higher levels of polyunsaturated FA of the latter might overcome the condensing effect of the head group. From Table 2 we infer that *C. vicinus* uses the polyunsaturated fatty acids present in PE whereas *D. magna* uses the molecular architecture of PC head groups to assure a comparable physical state of their membranes in warm temperatures.

The increase in PE level on exposure to cold, seen with both *C. vicinus* and *D. magna,* has also been noted with some other species (9, 24-26). Wieslander et al. (27) propose that the stability of the bilayers at reduced temperatures requires an increase in wedge-shaped PL (PE in this case) as well as an enhanced incorporation of polyunsaturated FA. The present results indicate (Tables 2 and 3) that *D. magna* only partially fulfills this requirement because it is unable to incorporate more polyunsaturated FA in PE when exposed to cold.

Comparison of Figures 1 and 2 indicates that *C. vicinus* completely adapts its PL transitional state to temperature but *D. magna* does not. The differences between the onset and completion of phase-separation temperatures exactly corresponded to the difference in the environmental temperature in the case of the former species. An additional proof that *C. vicinus* regulates the physical state of their phospholipids very precisely according to temperature comes from observations made on animals collected from waters at 15 C. These crustaceans kept the onset of phase-separation temperature at 14 C (data not shown). The adjustment of PL transitional state to temperature by *C. vicinus* can be expected to be as rapid as that given with the production of long-chain polyunsaturated FA when exposed to cold (13). Failure to regulate PL phasical state in *D. magna* might be one of the reasons why this species spends the cold season in the form of resting eggs. Membrane functions (permeability, kinetics of membrane bound enzymes and so forth) are known to be altered drastically below phase-separation temperature. This, and the instability of bilayers expected from the studies of Wieslander et al. (27), might render these structures unsuitable to fulfill normal functions in cold temperatures. As an imbalance in metabolism, as also suggested for plants sensitive to chilling (28), cannot be tolerated beyond certain limits, adaptation of PL physical state to temperature can be regarded as a requisite for the survival of crustaceans at reduced temperature.

We are aware that total PL, as investigated in this study, are representative of only average membranes, and that individual membranes (e.g., endoplasmic reticulum, mitochondria, plasma membranes) might differ in their phospholipid compositions and physical states. The response we found to a temperature decrease may be more pronounced in one type of membrane than in another. However, the individual membranes would seem likely to exhibit the same response to a temperature decrease as that observed with total PL.

REFERENCES

1. Sinensky, M. (1974) Proc. Natl. Acad. Sci. USA 72, 522-525.
2. Martin, Ch.E., Siegel, D., and Aarson, L.R. (1981) Biochim. Biophys. Acta 665, 299-407.
3. Janoff, A.S., Haug, A., and McGroarty, E.J. (1979) Biochim. Biophys. Acta 555, 56-66.
4. Dickens, B.F., and Thompson, G.A., Jr. (1981) Biochim. Biophys. Acta 644, 211-218.
5. Chapman, E., Wright, L.C., and Raison, J.K. (1979) Plant Physiol. 63, 363-366.

6. Furtado, D., Williams, W.P., Brain, A.P.R., and Quinn, P.J. (1979) Biochim. Biophys. Acta 555, 352-357.
7. Vigh, L., Horvath, I., Farkas, T., Horvath, L.I., and Belea, A. (1979) Phytochem. 18, 787-789.
8. Kallapur, V.L., Downer, R.G., George, J.C., and Thompson, J.E. (1982) F. Insect Biochem. 12, 115-212.
9. Cossins, A.R. (1977) Biochim. Biophys. Acta 470, 395-411.
10. McElhany, R.N. (1974) J. Mol. Biol. 84, 145-157.
11. Janoff, A.S., Gupta, S., and McGroarty, E.J. (1980) Biochim. Biophys. Acta 598, 641-644.
12. Farkas, T. (1979) Comp. Biochem. Physiol. 64B, 71-76.
13. Farkas, T., Kariko, K., and Csengeri, I. (1981) Lipids 16, 418-422.
14. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497-509.
15. Rouser, G., Fleischer, S., and Yamamoto, A. (1970) Lipids 5, 494-496.
16. Seelig, J. (1966) in Spin Labeling: Theory and Applications (Berliner, L.J., ed.) Vol. 1, pp. 373-409, Academic Press, New York.
17. Shimsick, E.J., and McConnel, H.H. (1973) Biochemistry 12, 2351-2360.
18. Mead, J.F. (1968) Progr. Chem. Fats Lipids 9, 161-192.
19. Chapelle, S., Zwingelstein, G., Meister, R., and Brichon, G. (1979) J. Exp. Zool. 210, 371-380.
20. Dickens, B.F., and Thompson, G.H., Jr. (1982) Biochemistry 21, 3604-3611.
21. Esser, A.F., and Sousa, K.A. (1975) Proc. Natl. Acad. Sci. USA 71, 4111-4115.
22. Huang, L., Lorch, S.K., Smith, G.G., and Haug, A. (1974) FEBS Letters 43, 1-5.
23. Phillips, M.C., Finer, E.G., and Hauser, M. (1972) Biochim. Biophys. Acta 290, 397-402.
24. Van Den Thillart, G., and De Bruin, G. (1981) Biochim. Biophys. Acta 713, 439-447.
25. Aaronson, L.R., Johnston, A.M., and Martin, Ch.E. (1982) Biochim. Biophys. Acta 713, 456-462.
26. Brichon, G., Chapelle, S., and Zwingelstein, G. (1980) Comp. Biochem. Physiol. 67B, 647-652.
27. Wieslander, A., Christiansen, A., Rilfors, L., and Lindbrom, G., Biochemistry 19, 3650-3655.
28. Lyons, J.M. (1973) Annu. Rev. Plant Physiol. 24, 445-456.

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Composition and Metabolism of Fatty Acids in Phospholipids of Density-Separated Red Cells of Rats

MANABU KUNIMOTO, KUNIMITSU KAYA and **TAKASHI MIURA**, *Basic Medical Sciences Division, The National Institute for Environmental Studies, Tsukuba, Ibaraki 305, Japan*

ABSTRACT

Fatty acid compositions of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) and the rates of fatty acid esterification to these phospholipids (PL) were measured in intact rat red cell populations of different ages separated by density gradient centrifugation in order to clarify changes in membrane lipids of red blood cells during in vivo aging. Fatty acid compositions of PC and PE altered progressively as red cells became denser. Changes in unsaturated fatty acids occurred predominantly at the 2-position of PC and PE and those in saturated fatty acids at both positions. The esterification rates of 5 major fatty acids decreased as red cells became denser and those of oleic acid, linoleic acid and arachidonic acid to both PC and PE of fraction I cells (oldest cells) were 37-51% those of fraction IV cells (youngest cells). Reduction in the rates of fatty acid esterification appeared to occur in the course of red cell maturation because reticulocyte-enriched cell fractions showed 4.5-14.5 times higher rates of linoleic acid and arachidonic acid esterifications to PC and PE.
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INTRODUCTION

Red blood cells circulate in the bloodstream for a definite period. Senescent cells are sequestered in the reticuloendothelial system (1,2). Changes in red cell components and their functions during survival in the bloodstream, in vivo aging, have been extensively studied using density-separated young and old cells. Old cells have been demonstrated to be denser and smaller in size (3), have a reduced deformability (4) and become osmotically fragile (5). A decrease in enzymatic activities has been also observed in the denser cells (6,7).

Changes in membrane lipids and their metabolism during in vivo aging of red blood cells have not been well confirmed. Alteration of polyunsaturated fatty acids has been demonstrated in cell membranes of old and young cells (8-10). Although reticulocytes are able to synthesize fatty acids and phospholipids (PL) de novo, matured mammalian erythrocytes cannot alter fatty acid chain length or degree of unsaturation, or synthesize PL de novo (11-13). Fatty acid metabolism of matured red cells is limited to deacylation of endogenous PL and reacylation of the resulting lysophospholipids and exchange of intact PL molecule with an exogenous one (14). In order to clarify changes in membrane lipids of red blood cells during in vovo aging, we measured the fatty acid compositions at the 1 and 2 positions of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) of density-separated red cells and the in vitro esterification rates of fatty acids to these PL.

MATERIALS AND METHODS

Chemicals

Radioactive fatty acids purchased from New England Nuclear (Boston, MA) were [5,6,8,9,11,12,14,15-^{3}H] arachidonic acid (78.2 Ci/mmol), [1-^{14}C] linoleic acid (56.5 mCi/mmol), [9,10-^{3}H] oleic acid (5.7 Ci/mmol), [1-^{14}C] stearic acid (55.7 mCi/mmol) and [9,10-^{3}H] palmitic acid (11.8 Ci/mmol). Other chemicals were purchased from the following companies: Boehringer Mannheim (Germany), phospholipase A_2 [EC 3. 1. 1. 4] from *Crotalus durissus terrificus;* Pharmacia Fine Chemicals (Piscataway, NJ), Dextran T40, lot. FL 18974; Wako Pure Chemicals (Tokyo, Japan), reagent kit for nonesterified fatty acid and phenylhydrazine. All other chemicals were of at least reagent grade.

Fractionation of Red Blood Cells

Blood samples were obtained from ether-anesthetized male Wistar rats (body weight: 400-500 g) using heparin as anticoagulant. The blood was mixed with 1/3 vol 50% Dextran T40, layered on the Dextran T40 discontinuous density gradient and centrifuged at 90,000 × *g* for 90 min at 4 C, using a Beckman L5-65B centrifuge with a swing rotor (SW 28, 40 ml × 6). The gradient was consisted of 30%, 32%, 34% (8 ml each) and 50% Dextran T40 solutions (3 ml). The Dextran T40 solutions were prepared according to the method of Abraham et al. (15). Four discrete zones of red blood cells were obtained and were designated as

fractions I-IV from the bottom. Red blood cells from each fraction were collected and washed 3 times with saline to remove Dextran. During this procedure, no visible lysis of red cells was observed. Contents of reticulocytes and leukocytes in each red-cell fraction were determined microscopically according to standard methods (16). Fraction IV cells contained 2-4% of reticulocytes, which were identified by brilliant cresyl blue staining with 1000 or more red cells counted to determine the percentage of reticulocytes. Three other fractions contained less than 0.1%. Content of leukocytes was less than 0.02% of the total cells in 4 fractions.

Preparation of Reticulocyte-Enriched Cell Fraction

Rats (450-500 g) were injected with phenylhydrazine hydrochloride for 3 consecutive days (30 mg/kg body weight/day). On day 7, blood samples were obtained. Fraction IV cells were prepared by density-gradient centrifugation and the reticulocyte counts were performed as described above.

Analysis of Membrane Lipids

Lipids were extracted by the method of Folch et al. (17) from red-cell membranes obtained according to the procedure of Dodge et al. (18). PC and PE were isolated by thin layer chromatography [HP-TLC silica-gel plates, E. Merck; chlorform/methanol/28% ammonium/water (73:27:2:3, v/v/v/v)] and were heated with boron trifluoride in methanol by the method of Metcalfe and Schmitz (19). Resulting fatty acid (FA) methylesters were analyzed at 230 C in a Shimadzu GC-7AG gas chromatograph (GC) (Shimadzu Corp., Japan) using a flame ionization detector (FID) and a glass column (4.1 m × 0.5 cm) packed with 5% Shinchrom E-71 (nitrile polyester) on Shimalite (AW) (80-100 mesh, diatomaceous earth) (Wako Pure Chemicals). In this condition, 16:0 and 16:1 were not always separated clearly so the percentage of 16:0 + 16:1 was calculated. Areas of peaks and percent composition of FA methylesters were computed using a Shimadzu Chromatopack R1-A Data System (Shimadzu Corp.).

The positional distribution of FA in PC and PE was analyzed by treatment with snake venom phospholipase A_2 as described by Ferber et al. (20). PL (0.5-1.0 μmol), dissolved in 2 ml of diethylether/ethanol (19:1, v/v) containing 1.5 mM butylated hydroxytoluene, were mixed with 100 μl of 0.2 M Tris-HCl (pH 8.0), 100 μl of enzyme solution (1 mg/ml) and 50 μl of 0.2 M $CaCl_2$ and incubated at 30 C with vigorous shaking for 6 hr. Then the lipids were recovered and separated by TLC (chloroform/methanol/water [65:25:4, v/v/v]). More than 98% of PC and 93% of PE were hydrolyzed. Resulting free fatty acids (FFA) and lysophospholipids were recovered and their fatty acid compositions were examined by gas liquid chromatography (GLC).

The plasma was isolated from heparinized rat blood by centrifugation. Lipids were extracted from plasma, and FFA and PC were separated from extracted lipids by TLC. The composition of FA was determined by GLC as described above.

Esterification of Radioactive Fatty Acids to Phospholipid of Density-Separated Red Cells

Plasma was heated at 56 C for 1 hr to destroy lecithin-cholesterol acyltransferase (21) and mixed with radioactive FA. When 1 μCi of radioactive FA was added to 1 ml of plasma, the specific radioactivity in plasma was as follows: palmitic acid, 33.2 μCi/μmol; stearic acid, 36.9 μCi/μmol; oleic acid, 45.9 μCi/μmol; linoleic acid, 19.6 μCi/μmol; and arachidonic acid, 192.3 μCi/μmol. The plasma containing radioactive FA was preincubated at 37 C for 30 min. A 1.3 ml aliquot of the plasma containing 1.3 μCi of radioactive FA was added to 1 ml of packed red cells and incubated at 37 C with gentle shaking. In the absence of red blood cells, radioactive FA were not esterified to plasma PL in the whole period of incubation. Two hours after incubation, cells were centrifuged, washed 3 times with saline and hemolyzed. Lipids were extracted from red-cell membranes and PC and PE were separated from extracted lipids by TLC. The radioactivities of PC and PE were determined using a liquid scintillation counter (Packard model 3325). The amount of inorganic phosphorus was determined as described by Gerlach and Deuticke (22). The FA esterification to PC and PE of the reticulocyte-enriched cell fraction was examined by incubation with plasma obtained from untreated rats in the presence of radioactive FA.

RESULTS

Fatty Acid Compositions of PC and PE of Density-Separated Red Cells

Table 1 shows the FA composition of PC of whole and fractionated red cells. Significant alterations in FA composition were observed between fraction IV and fraction I cells. The percentages of 18:0 and 18:2 increased as red cells became denser from fractions IV to I,

TABLE 1

Fatty Acid Composition of PC in Density-Separated Red Cells[a]

		Fatty acid composition (%)		
		Whole cells	Fraction IV	Fraction I
16:0+16:1[b]	Overall	46.9 ± 0.7	50.4 ± 1.5	45.4 ± 0.6***
	1 Position	54.2 ± 2.4	58.0 ± 2.8	53.5 ± 0.8*
	2 Position	36.3 ± 4.5	36.5 ± 3.1	32.1 ± 5.2
18:0	Overall	14.0 ± 0.7	11.3 ± 0.6	16.9 ± 0.4***
	1 Position	28.0 ± 2.1	22.7 ± 0.7	32.5 ± 1.2***
	2 Position	5.2 ± 0.4	4.0 ± 0.7	6.9 ± 0.5*
18:1	Overall	8.7 ± 0.2	10.5 ± 0.5	7.2 ± 0.2***
	1 Position	7.7 ± 0.9	8.6 ± 0.6	7.7 ± 0.6
	2 Position	10.1 ± 0.8	12.2 ± 0.4	8.8 ± 1.1**
18:2	Overall	16.9 ± 0.6	15.4 ± 0.5	18.6 ± 0.7***
	1 Position	6.2 ± 0.9	6.6 ± 0.5	6.3 ± 1.3
	2 Position	24.3 ± 1.2	23.8 ± 1.2	29.5 ± 1.7**
20:4	Overall	10.7 ± 0.8	10.9 ± 1.1	10.0 ± 0.8
	1 Position	3.9 ± 2.1	4.1 ± 1.9	19.1 ± 2.4
	2 Position	20.6 ± 3.3	20.1 ± 2.9	19.1 ± 2.4
22:6	Overall	1.7 ± 0.2	1.5 ± 0.3	1.7 ± 0.2
	1 Position	—	—	—
	2 Position	2.7 ± 0.5	2.4 ± 0.1	2.7 ± 0.6

[a]Fatty acid compositions at the 1 and 2 positions of PC in unfractionated (whole) and density-separated (fractions IV and I) red cells were determined as described in Materials and Methods. Fatty acid compositions of unhydrolyzed PC (overall) were also determined. The values given were mean ± SD (n=6 for overall, n=4 for 1 and 2 positions). The difference between fraction IV and I cells was tested by student's *t*-test: *$p<0.05$, **$p<0.01$, ***$p<0.001$.

[b]Ca. 98% of these fatty acids were 16:0.

whereas 16:0 + 16:1 and 18:1 decreased. Changes in unsaturated FA occurred predominantly at the 2 position and those in saturated FA at both positions. Alterations of FA composition of PE were somewhat different from those of PC (Table 2). The percentages of 18:0, 18:1 and 18:2 increased significantly as red cells became denser from fractions IV to I. By contrast, those of 16:0 + 16:1, 20:4, 22:5 and 22:6 decreased. Changes in saturated FA were mainly caused by changes at the 1 position. On the other hand, changes in unsaturated FA were mainly caused by changes at the 2 position. These alterations of FA compositions of PC and PE were progressively more marked as red cells became denser (data not shown).

Esterification of Radioactive Fatty Acids to PC and PE of Density-Separated Red Cells

The rat plasma contained 0.11 mEq FFA, which was comprised of 27.3%, 16:0; 8.3%, 18:0; 19.6%, 18:1; 30.1%, 18:2; 4.6%, 20:4; 10.1% minor FA. The addition of radioactive FA caused increases in the concentrations of plasma FA as follow: 18:0, 0.009-0.027 mEq; 18:2, 0.033-0.051 mEq. Increases in the concentrations of other FA were less than 0.2% of the original plasma ones. When red blood cells were incubated with plasma containing radioactive FA, esterifications of radioactive FA to PC and PE of red cell membranes continued linearly for at least 2 hours. Table 3 shows the esterification rates of radioactive FA to PC and PE of red cell membranes. The esterification rates of all FA tested to PC and PE decreased as red cells became denser from fractions IV to III. The esterifications of 18:1, 18:2 and 20:4 to PC of fraction III cells were 51%, 45% and 60% those of fraction IV cells, respectively. Also, the esterifications of 18:1, 18:2 and 20:4 to PE of fraction III cells were 51%, 45% and 65% those of fraction IV cells. Among denser fractions III, II and I, no significant difference was observed.

Esterification of Radioactive Fatty Acids to PC and PE of Reticulocyte-Enriched Red Cell Fraction

Fraction IV cells comprised the youngest erythrocytes and a small number of reticulocytes. In order to examine possible involvement of reticulocytes in higher esterification rates of fraction IV cells, reticulocyte-enriched blood

TABLE 2

Fatty Acid Composition of PE in Density-Separated Red Cells[a]

		Fatty acid composition (%)		
		Whole cells	Fraction IV	Fraction I
16:0+16:1[b]	Overall	27.7 ± 0.7	29.0 ± 0.5	25.5 ± 0.9***
	1 Position	57.3 ± 2.1	60.4 ± 2.9	52.2 ± 2.1**
	2 Position	4.9 ± 1.1	3.8 ± 1.9	6.7 ± 0.6*
18:0	Overall	3.8 ± 0.2	3.3 ± 0.1	4.3 ± 0.5**
	1 Position	9.9 ± 1.1	8.6 ± 1.4	12.2 ± 2.2
	2 Position	1.4 ± 0.8	0.7 ± 0.1	1.6 ± 0.7
18:1	Overall	10.8 ± 0.2	9.1 ± 0.6	13.6 ± 0.6***
	1 Position	21.0 ± 1.0	19.3 ± 2.2	21.9 ± 3.6
	2 Position	5.7 ± 0.6	5.2 ± 1.2	7.8 ± 0.5*
18:2	Overall	6.1 ± 0.3	4.0 ± 0.4	9.2 ± 1.0***
	1 Position	5.9 ± 0.6	5.7 ± 1.7	7.6 ± 2.3
	2 Position	7.1 ± 1.3	5.3 ± 0.4	12.9 ± 0.5***
20:4	Overall	34.9 ± 0.6	36.7 ± 1.2	31.9 ± 1.5***
	1 Position	5.5 ± 1.7	6.2 ± 4.0	6.0 ± 1.7
	2 Position	55.8 ± 3.0	57.6 ± 1.3	48.1 ± 2.7**
22:4	Overall	3.6 ± 0.1	3.9 ± 0.3	3.3 ± 0.3
	1 Position	—	—	—
	2 Position	5.2 ± 0.5	5.8 ± 0.6	4.4 ± 0.7*
22:5	Overall	6.5 ± 0.1	6.9 ± 0.4	5.9 ± 0.2***
	1 Position	—	—	—
	2 Position	9.6 ± 0.7	10.9 ± 1.1	8.7 ± 0.7*
22:6	Overall	5.9 ± 0.3	6.2 ± 0.1	5.3 ± 0.2***
	1 Position	—	—	—
	2 Position	8.6 ± 0.2	9.1 ± 0.4	7.7 ± 0.5**

[a]Fatty acid compositions at the 1 and 2 positions of PE in unfractionated (whole) and density-separated (fractions IV and I) red cells were determined as described in Materials and Methods. Fatty acid compositions of unhydrolyzed PE (overall) were also determined. The values given were mean ± SD (n=6 for overall, n=4 for 1 and 2 positions). The difference between fraction IV and I cells was tested by student's *t*-test: *$p<0.05$, **$p<0.01$, ***$p<0.001$.

[b]Ca. 70% of these fatty acids were 16:0.

TABLE 3

Esterification Rates of Radioactive Fatty Acid from Plasma to PC and PE of Density-Separated Red Cells[a]

	Incorporation rate of fatty acid (μmol/mol phospholipid/2 hr)				
	Whole cells	Fraction IV	Fraction III	Fraction II	Fraction I
Phosphatidylcholine					
16:0 (2)	103 ± 7	145 ± 4	113 ± 14	100 ± 12	121 ± 26
18:0 (2)	14 ± 1	18 ± 4	16 ± 1	11 ± 1	12 ± 1
18:1 (4)	53 ± 12	145 ± 3*	61 ± 1	51 ± 5	54 ± 7
18:2 (8)	204 ± 39	515 ± 120**	234 ± 44	197 ± 25	222 ± 28
20:4 (6)	139 ± 43	273 ± 79*	164 ± 35	133 ± 28	114 ± 10
Phosphatidylethanolamine					
16:0 (2)	21 ± 1	26 ± 0	17 ± 0	19 ± 3	23 ± 4
18:0 (2)	7 ± 1	10 ± 1	n.d.	5 ± 1	5 ± 1
18:1 (4)	37 ± 10	85 ± 10**	43 ± 4	35 ± 6	32 ± 4
18:2 (8)	60 ± 14	134 ± 37***	60 ± 12	53 ± 9	58 ± 9
20:4 (6)	56 ± 8	91 ± 11*	59 ± 10	56 ± 11	46 ± 3

[a]Esterification of radioactive fatty acids to PC and PE of unfractionated (whole) and density-separated (fraction IV to I) red cells was examined as described in Materials and Methods. The values given were mean ± SD. The number of the experiment was given in parentheses. The difference between fraction IV and III cells was tested by student's *t*-test: *$p<0.05$, **$p<0.01$, ***$p<0.001$.

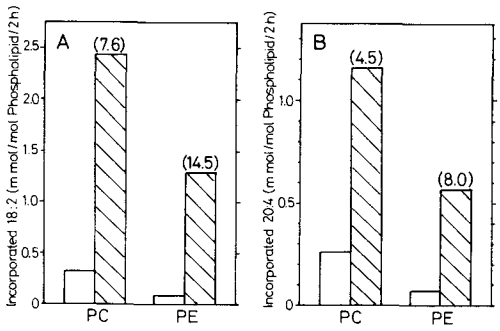

FIG. 1. Fatty acid esterification to PC and PE of fraction IV cells obtained from phenylhydrazine-treated rats. Esterification of 18:2(A) and 20:4(B) to PC and PE of fraction IV cells prepared from bloods of phenylhydrazine-treated (hatched bars) and untreated rats (open bars) were examined as described in Materials and Methods. The values represented the mean of 2 experiments and the ratios of phenylhydrazine-treated to untreated were shown in parentheses.

was obtained from phenylhydrazine-treated rats and fractionated by density-gradient centrifugation. Reticulocytes were located exclusively in fraction IV and accounted for more than 40% of fraction IV cells. Esterification of radioactive FA to fraction IV cells of phenylhydrazine-treated rats was compared with that of fraction IV cells of untreated rats (Fig. 1). Esterification rates of 18:2 and 20:4 to PE was 8.0-fold and 14.5-fold as high, respectively, in phenylhydrazine-treated rats as in untreated ones. A similar result was obtained for esterification of these FA to PC.

DISCUSSION

In total lipids of human red cells, an increase in 18:2 and a decrease in 20:4 were found to accompany red cell aging (8,9). Similar changes have been demonstrated in rat red cells (10). The present results confirmed that alterations in FA compositions of PC and PE, major PL of rat red cells, proceeded progressively during in vovo aging of rat red cells (Tables 1 and 2). Changes in unsaturated fatty acids occurred predominantly at the 2 position of PC and PE and those in saturated FA at both positions.

In vitro studies using density-separated red cells have shown that the rates of esterification of radioactive FA from plasma to cell membrane PC and PE decreased to varying degrees depending on the FA species at the earliest stage of red cell aging (Table 3). This reduction appears to occur in the course of maturation of reticulocytes to erythrocytes because the reticulocyte-enriched red-cell fraction obtained from phenylhydrazine-treated rats showed markedly higher rates of FA esterification (Fig. 1). Based on the data of Figure 1 and Table 3, the esterification rates of phenylhydrazine-induced reticulocytes were calculated. Reticulocyte-enriched red-cell fraction contained 40% reticulocytes and 60% matured red cells, and esterified 18:2 and 20:4 to PE at the rates of 1.29 and 0.57 mmol/2 hr, respectively. If the esterification rates of matured red cells are supposed to be those of whole cells (Table 3), the esterification rates of 18:2 and 20:4 to PE in phenylhydrazine-induced reticulocytes are estimated at 3.13 and 1.35 mmol/2 hr, respectively. Using the esterification rates estimated for phenylhydrazine-induced reticulocytes and those of whole cells, the esterification rates of fraction IV cells obtained from untreated rats, which contained 2-4% reticulocytes, were calculated and proved to be 0.122-0.183 and 0.082-0.108 mmol/2 hr for 18:2 and 20:4 to PE, respectively. These values are consistent with the observed ones of fraction IV cells obtained from untreated rats (Table 4). Similarly, the esterification rates of 18:2 and 20:4 to PC in fraction IV cells were calculated at 0.317-0.429 and 0.193-0.247 mmol/2 hr, respectively, based on the esterification rates of phenylhydrazine-induced reticulocytes (5.83 and 2.85 mmol/2 hr for 18:2 and 20:4, respectively). These results support the contention that the higher rates of FA esterification of fraction IV cells obtained from untreated rats are mainly caused by reticulocytes present in this fraction. Three denser fractions, III, II and I, contained less than 0.1% of reticulocytes. Participation of reticulocytes in the FA esterification of these 3 fractions seems to be less than 6% of the total esterification of 18:2 and 20:4 to PE and PC.

Matured mammalian erythrocytes cannot synthesize PL de novo and their FA metabolism is limited to the deacylation-reacylation reaction, which is catalyzed by phospholipase A_2, acyl CoA lipase and acyl CoA-lysophospholipid acyltransferase (14,23). On the other hand, reticulocytes are able to synthesize PL de novo (11-13) so that plasma FFA are esterified to PC and PE of reticulocytes by both de novo synthesis and the deacylation-reacylation reaction. The higher rates of FA esterification to PC and PE of fraction IV cells are probably caused by de novo synthesis of PL performed by reticulocytes present in this fraction. In the course of reticulocyte maturation, the FA esterification to PL will decrease by the loss of de novo synthesis and the decreased rate of FA esterification, which is performed by the deacylation-

reacylation system, is maintained throughout the subsequent process of red cell aging (Table 3). FA compositions of PC and PE of red-cell membranes still altered progressively during in vivo aging of mature red cells. The deacylation-reacylation reaction is conceivably closely related to changes in the FA composition of PL during in vivo aging of erythrocytes.

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REFERENCES

1. Cooper, R.A., and Shattil, S.J. (1971) New Engl. J. Med. 285, 1514-1520.
2. Weed, R.I. (1970) Amer. J. Med. 49, 147-150.
3. Piomelli, S., Lurinsky, G., and Wasserman, L.R. (1967) J. Lab. Clin. Med. 69, 659-674.
4. Weed, R.I., La Celle, L., and Merrill, E.W. (1969) J. Clin. Invest. 48, 795-809.
5. Marks, P.A., and Johnson, A.B. (1958) J. Clin. Invest. 37, 1542-1548.
6. Cohen, N.S., Ekholm, J.E., Luthra, M.G., and Hanahan, D.J. (1976) Biochim. Biophys. Acta 419, 229-242.
7. Seaman, C., Wyss, S., and Piomelli, S. (1980) Am. J. Hematol. 8, 31-42.
8. Van Gastel, C., Van Den Berg, D., De Gier, J., and Van Deenen, L.L.M. (1965) Br. J. Haematol. 11, 193-199.
9. Phillips, G.B., Dodge, J.T., and Howe, C. (1969) Lipids 4, 544-549.
10. Walker, B.L., and Yurkowski, M. (1967) Biochem. J. 103, 218-224.
11. Pittman, J.G., and Martin, D.B. (1966) J. Clin. Invest. 45, 165-172.
12. Percy, A.K., Schmell, E., Earles, B.J., and Lennarz, N.J. (1973) Biochemistry, 12, 2456-2461.
13. Ballas, S.K., and Burka, E.R. (1974) Biochim. Biophys. Acta 337, 239-247.
14. Shohet, S.B. (1977) in Lipid Metabolism in Mammals, Vol. I, (Snyder, F., ed.) pp. 189-208, Plenum Press, New York.
15. Abraham, E.C., Walker, D., Gravely, M., and Huisman, T.H.J. (1975) Biochem. Med. 13, 56-77.
16. Miale, J.B. (1977) in Laboratory Medicine Hematology, pp. 998-1000, the C.V. Mosby Company, St. Louis, MO.
17. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497-509.
18. Dodge, J.T., Mitchell, C., and Hanahan, D.J. (1963) Arch. Biochem. Biophys. 110, 119-130.
19. Metcalfe, L.D., and Schmitz, A.A. (1961) Anal. Chem. 33, 363-364.
20. Ferber, E., De Pasquale, G.G., and Resch, K. (1975) Biochim. Biophys. Acta 398, 364-376.
21. Renooij, W., Van Golde, L.M.G., Zwaal, R.F.A., and Van Deenen, L.L.M. (1976) Eur. J. Biochem. 61, 53-58.
22. Gerlach, E. and Deuticke, B. (1963) Biochem. Z. 337, 477-479.
23. Dise, C.A., Goodman, D.B.P., and Rasmussen, H. (1980) J. Lipid Res. 21, 292-300.

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Effects of Triarimol, Tridermorph and Triparanol on Sterol Biosynthesis in Carrot, Tobacco and Soybean Suspension Cultures

GLENN HOSOKAWA,[a] GLENN W. PATTERSON,[a] and WILLIAM R. LUSBY,[b]
[a]*Department of Botany, University of Maryland, College Park, MD 20742 and*
[b]*Insect Physiology Laboratory, Beltsville Agricultural Research Center, Beltsville, MD 20705*

ABSTRACT

The effects of triarimol, tridemorph and triparanol on sterol biosynthesis in carrot, tobacco and soybean suspension cultures were studied. The 3 plant species normally contain campesterol, stigmasterol and sitosterol as major sterols. Triarimol inhibited demethylation at C 14 and the second alkylation of the side chain in all 3 species. The primary effects of tridemorph were the inhibition of the opening of the 9β,19-cyclopropane ring and the second alkylation of the side chain. Triparanol treatments resulted in the accumulation of 14α-methyl sterols, and the inhibition of second alkylation in the side chain in carrot and tobacco cultures. Cyclopropyl sterols also accumulated in carrot and tobacco cultures treated with triparanol. Triparanol did not alter the sterol composition of soybean cultures except for decreasing concentrations of campesterol and stigmasterol and increasing amounts of sitosterol.
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INTRODUCTION

Numerous inhibitors of sterol metabolism have been used in studies of sterol biosynthesis. A fungicide, triarimol [α-(2,4-dichlorophenyl)-α-phenyl-5-pyrimidine-methanol], is known to inhibit 14α-demethylase in animal tissues (1), in several species of water molds (Oomycetes) (2,3), in *Ustilago maydis* (4,5) and in *Chlorella* (6). Other inhibitory sites of triarimol include $\Delta^{24(28)}$-reductase (4,5), the synthesis of Δ^{22}-sterols (4,5,6) and the second alkylation at C 24 (6). In bean seedlings *(Phaseolus vulgaris)*, triarimol retards growth but does not appear to affect sterol biosynthesis (7).

Fenarimol [α-(2-chlorophenyl)-α-(4-chlorophenyl)-5-pyrimidine-methanol], a compound structurally similar to triarimol, inhibits the $\Delta^{24(28)}$-reductase and the 14α-demethylase in suspension cultures of bramble *(Rubus fruticosus)* (8). In this higher plant cell system, fenarimol blocks the synthesis of normally occurring Δ^5-sterols and stimulates the accumulation of sterols with 14α-methyl and $\Delta^{5,24(28)}$-unsaturation.

Another fungicide, tridemorph (2,6-dimethyl-N-tridecyl-morpholine), has been shown to interfere with ergosterol synthesis in fungi studied (9,10), and is a specific inhibitor of the $\Delta^8 \rightarrow \Delta^7$ isomerase. The accumulation of sterols having a Δ^8-bond was also observed in water molds treated with tridemorph (2,3). Zymosterol, fecosterol $\Delta^{8,24(28)}$-ergostadienol and stigmasta-8,E-24(28)-dienol were the principal Δ^8-sterols accumulated in tridemorph-treated water molds. In 2 higher plant systems, bramble suspension cultures (11) and corn seedlings (12), tridemorph effectively blocks cycloeucalenol-obtusifoliol isomerase, the enzyme involved in opening the 9β,19-cyclopropane ring.

A hypocholesterolemic compound, triparanol (1-[4(diethylaminoethoxy)-pehnyl]-1-(p-tolyl)-2-(p-chlorophenyl)-ethanol), inhibits the reduction of desmosterol to cholesterol in animal systems (13,14). The accumulation of desmosterol in an insect (15) and a nematode (16) results as triparanol blocks the conversion of sitosterol to cholesterol. In *Saccharomyces cerevisiae*, triparanol inhibits the $\Delta^8 \rightarrow \Delta^7$ isomerase and interferes with the conversion of lanosterol to ergosterol (17,18). In 3 species of *Chlorella* studied, triparanol inhibits $\Delta^8 \rightarrow \Delta^7$ isomerase (19,20,21), Δ^7 and Δ^{14} reductase (19,20), the second alkylation at C 24 (19,20, 21), 14α-demethylase (19,20,21,22) and the synthesis of Δ^{22}-sterols (19), depending on the *Chlorella* species.

The effects of sterol inhibitors have been extensively studied in animals, fungi and algae. With the exception of recent work with bramble and corn (11,12), very little published data is available about the effects of these compounds on sterol biosynthesis in higher plants. Suspension cultures of carrot, tobacco and

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soybean were used to determine the effects of triarimol, tridemorph and triparanol on sterol biosynthesis of these agriculturally important plants.

MATERIALS AND METHODS

Suspension cultures of carrot, *Daucus carota* var. Danvers, were grown in Murashige-Skoog media (23) supplemented with 1 mg/l, 2,4-D, 0.1 mg/l Benzylamino purine and 30 g/l sucrose. Cultures of soybean, *Glycine max* var. Acme, were grown in R3 media composed of LS salts (24) supplemented with 0.5 mg/l thiamine-HCl, 5 mg/l Indole acetic acid, 0.3 mg/l kinetin, 0.5 mg/l 2,4-D and 40 g/l sucrose. Suspension cultures of tobacco, *Nicotiana tabacum* var. Sulfur, were grown in Murashige-Skoog media to which 2 mg/l naphtalene acetic acid, 0.1 mg/l kinetin and 30 g/l sucrose were added as a supplement. Cultures were grown under fluorescent light (24 hr) at 11,195 Lux. Suspension cultures (150 ml) were maintained at room temperature (26 C) with constant shaking at 140 rpm.

Triarimol, tridemorph and triparanol sterilized in a filter were added in solution (in ethanol) to culture media. Cultures were treated with various levels of inhibitors designed to produce ca. 50% growth inhibition (Table 1). Controls were used for each species with and without ethanol. At a logarithmic phase of the growth cycle, control cells were harvested after the fifth day and treated cells were harvested after the eighth day of treatments. Each treatment was repeated at least 3 times for each species.

Freeze-dried cells were refluxed in a Soxhlet (24 hr) using chloroform/methanol (2:1, v/v). The crude lipid was saponified with 20% KOH in 80% EtOH (45 min) and the nonsaponifiable fraction was extracted with ether. The remaining aqueous fraction in the liquid-liquid extractor was acidified with 6N HCl, and reextracted with ether using liquid-liquid extractor (24 hr). The second ether fraction was collected, evaporated and the fatty acids esterified with a BCl_3-MeOH solution. The esterified fatty acid fraction was partitioned into hexane. Sterols were isolated from this fraction as well as the nonsaponifiable fraction by chromatography on alumina.

Sterols were separated into dimethyl, monomethyl and desmethyl sterol fractions by modification of the thin layer chromatography (TLC) described by Tu et al. (25).

Separation of unsaturated sterols (as acetates) was carried out using argentation TLC described by Vroman and Cohen (26).

Sterols were tentatively identified by gas liquid chromatography (GLC) (27) with a Varian 3700 gas chromatograph fitted with a 1.8 x 3.4 mm column of 3% SE-30 on Gas Chrom Q. Peak area calculations were processed with a Varian CDS 111 Chromatographic Data System.

Mass spectra (MS) were obtained using the method described by Berg et al. (3). MS of samples were compared with MS of authentic standards, and sterols were identified by GLC-MS (28).

TABLE 1

Comparison of Lipid Data from Control and Drug Treated Cultures of Carrot, Tobacco and Soybean[a]

Culture	Treatment	Dry wt. (g)	Total lipid (mg/g dry wt.)	Total sterol (mg/g dry wt.)
Carrot	Control	10.2(9.1-10.9)	107.6(101.1-115.9)	1.0(0.9-1.2)
	Triarimol (5 mg/l)	4.2(3.9-4.7)	112.2(104.4-120.3)	0.7(0.7-0.8)
	Tridemorph (3 mg/l)	2.3(2.1-2.7)	310.4(302.2-322.6)	1.4(1.2-1.7)
	Triparanol (10 mg/l)	5.4(4.9-6.1)	87.2(83.5-91.9)	1.8(1.6-2.2)
Tobacco	Control	16.0(15.0-17.3)	47.5(45.9-49.2)	1.3(1.2-1.4)
	Triarimol (10 mg/l)	12.5(11.7-13.0)	24.7(22.6-28.0)	0.7(0.5-0.8)
	Tridemorph (1 mg/l)	14.4(14.1-15.0)	15.2(13.9-16.8)	0.6(0.5-0.6)
	Triparanol (5 mg/l)	10.6(9.6-11.9)	48.3(46.1-51.2)	1.8(1.7-2.0)
Soybean	Control	22.9(21.8-24.4)	22.2(20.4-24.5)	1.0(0.8-1.1)
	Triarimol (10 mg/l)	8.0(7.3-8.9)	57.7(56.1-60.7)	1.1(0.9-1.2)
	Tridemorph (5 mg/l)	9.6(9.2-10.5)	45.6(43.5-48.4)	1.2(1.1-1.3)
	Triparanol (16 mg/l)	16.2(15.5-16.6)	50.0(48.8-52.1)	1.3(1.2-1.4)

[a]Values given are means of 3 replications with ranges in parentheses.

RESULTS AND DISCUSSION

Growth was inhibited as measured by dry-matter production in carrot, tobacco and soybean cultures when grown in media supplemented with either triarimol, tridemorph or triparanol (Table 1). The quantitites of total lipid and total sterol produced varied according to treatment with cultures of carrot and soybean, but increased in all treated soybean cultures compared to controls (Table 1).

The 3 major sterols of each control culture were campesterol, stigmasterol and sitosterol. A minute quantity of cholesterol was found in all 3 control cultures and a trace of 24-methylenecycloartanol in carrot and tobacco control cultures. The sterols from controls with and without ethanol were identical in each culture. As noted in Table 2, with the exception of 24-methylenecycloartanol, all the sterols in control cultures were Δ^5-sterols, accounting for 98% or more of total sterol. In other tissue cultures of higher plants campesterol, stigmasterol and sitosterol were the major sterols, and sitosterol was the predominant sterol (29-33).

Effects of Triarimol on Sterol Composition

Triarimol effectively altered the composition of sterols in carrot, tobacco and soybean cultures. All 3 cell cultues produced 14α-methyl-5α-ergost-8-en-3β-ol (Table 2). The spectrum was similar to that of the authentic compound as described by Doyle et al. (34). Triarimol also caused the accumulation of 14α-methyl-5α-ergost-8-en-3β-ol in *Chlorella* species (6). Obtusifoliol (4,14-dimethyl-5α-ergosta-8,24(28)-dien-3β-ol) was also detected in all cell cultures treated with the fungicide (Table 2). A large quantity of this sterol was present in the treated cultures of carrot and tobacco. The MS of the sterol matched the spectrum of authentic samples and was similar to the spectrum of obtusifoliol described by Ragsdale (4) and of *Chlorella* species (6) treated with triarimol.

A small quantity of cycloeucalenol [4,14-dimethyl-9β, 19-cyclo-5α-ergost-24(28)-en-3β-ol] accumulated only in carrot cultures treated with triarimol (Table 2). The MS was similar to the spectrum of cycloeucalenol described by Doyle et al. (22). A minute amount of 5α-stigmast-7-en-3β-ol was found in the cultures of carrot treated with triarimol. The MS of the sterol was similar to the spectrum of an authentic sample and similar to the one described by Doyle et al. (22).

The cultures of carrot and soybean (Table 2) produced 24-dihydroobtusifoliol (4,14-dimethyl-5α-ergost-8-en-3β-ol) when treated with the fungicide. The MS of the sterol was similar to that of the authentic sample and of the MS of 24-dihydroobtusifoliol from *C. emersonii* treated with triparanol (22). In *C. ellipsoidea* and *C. emersonii,* 24-dihydroobtusifoliol accumulated when treated with triarimol (6).

The remaining sterols in cultures treated with triarimol were sterols normally found in control cultures (Table 2). GLC-MS of these sterols indicated identity with campesterol, stigmasterol and sitosterol. However, all 3 were considerably reduced (μg/g dry wt.) in triarimol-treated cultures.

Triarimol appears to induce 2 principal effects on carrot, tobacco and soybean cultures. The fungicide caused a large accumulation of sterols with a 14α-methyl group (Table 3). The accumulation of sterols with a 14α-methyl group was noted in animals (1), water molds (2,3), *U. maydis* (4,5) and in *Chlorella* species (6) as a result of treatments with the fungicide. In these organisms, triarimol effectively blocked the removal of the 14-methyl group. The accumulation of sterols, e.g., 14α-methyl-5α-ergost-8-en-3β-ol, obtusifoliol, 24-dihydroobtusifoliol and 14α-methyl-5α-stigmast-8-en-3β-ol, indicates that triarimol is also an effective inhibitor of the 14α-demethylase in cultures of carrot, tobacco and soybean.

The other principal effect of triarimol on the 3 cultures is the inhibition of the second alkylation at C 24. Reduction of sterols with a 10-carbon side chain and an increase in the level of sterols having a 9-carbon side chain in triarimol-treated cultures were significant (Table 3). Compared with control cultures, the quantity of 24-methylene sterols increased from a trace to ca. 33% of total sterol in carrot, and increased to 30% of total sterol in tobacco (Table 3) cultures treated with triarimol. Sterols with 24-methylene groups are known to be precursors for the second alkylation at C 24 (35). The increase in sterols with a 9-carbon side chain, in 24-methylene sterols and the decrease in sterols with a 10-carbon side chain indicate that triarimol is an effective inhibitor of the second alkylation at C 24 in carrot, tobacco and soybean cells. The inhibition of the second alkylation was also observed in *Chlorella* species treated with triarimol (6).

The concentration of triarimol effective for the accumulation of 14α-methyl sterols varied with the organism studied. In fungi, the concentrations of triarimol effective in the accumulation of 14α-methyl sterols were 2 mg/l in *U. maydis* (4,5) and 10 mg/l in Oomycetes (2,3). Cultures of carrot, tobacco and soybean required 5 mg/l, 10 mg/l and 10 mg/l triarimol, respectively, for an effective inhibition of the C 14 demethylation. Therefore, these higher

TABLE 2

Comparison of Sterol Content from Control and Treated Cultures of Carrot, Tobacco and Soybean (μg/g dry weight)

Sterol	Carrot				Tobacco				Soybean			
	Control	Triarimol	Tridemorph	Triparanol	Control	Triarimol	Tridemorph	Triparanol	Control	Triarimol	Tridemorph	Triparanol
Cholesterol	11	5	11	5	7	6	8	–	1	–	–	–
Campesterol	213	194	228	185	370	116	72	174	212	74	54	206
Stigmasterol	352	104	285	176	300	206	73	168	361	106	6	327
Sitosterol	408	127	334	268	651	100	59	206	392	167	448	754
24-Methylenecholesterol	–	–	–	–	–	–	5	–	–	–	–	–
14α-Methyl-5α-ergost-8-en-3β-ol	–	27	–	194	–	41	–	214	–	572	92	–
24-Methylenepollinastanol	–	–	54	79	–	–	227	–	–	–	–	–
Obtusifoliol	–	194	–	17	–	198	–	239	–	10	–	–
24-Dihydroobtusifoliol	–	6	–	–	–	–	–	26	–	25	–	–
24-Methylpollinastanol	–	–	–	176	–	–	25	–	–	–	552	–
14α-Methyl-5α-stigmast-8-en-3β-ol	–	–	–	416	–	–	–	496	–	74	–	–
28-Isofucosterol	–	–	21	–	–	–	33	–	–	–	–	–
Cycloeucalenol	–	13	266	39	–	–	83	230	–	–	38	–
24-Methylenecycloartanol	6	37	165	210	–	–	–	13	–	–	2	–
Other sterols	–	23	16	55	–	–	–	26	–	42	18	–
Total	990	730	1380	1820	1328	667	585	1792	966	1070	1210	1287

TABLE 3

Comparison of Different Sterol Features Occurring in Control and Treated Cultures of Carrot, Tobacco and Soybean[a]

Sterol Feature	Carrot				Tobacco				Soybean			
	Control	Triarimol	Tridemorph	Triparanol	Control	Triarimol	Tridemorph	Triparanol	Control	Triarimol	Tridemorph	Triparanol
24-Methylene	1	33	33	19	–	30	54	27	–	1	3	–
4,4',14-Trimethyl	1	5	12	12	–	–	–	1	–	–	–	–
4,14-Dimethyl	–	29	17	3	–	30	14	28	–	3	3	–
4,14-Dimethyl $\Delta^{8(9)}$	–	28	–	1	–	30	–	15	–	3	–	–
14α-Methyl $\Delta^{8(9)}$	–	4	–	34	–	6	–	40	–	60	8	–
9,19-Cyclopropane	1	7	33	28	–	–	58	14	–	–	49	–
$\Delta^{5,24(28)}$	–	–	2	–	–	–	7	–	–	–	–	–
Δ^{5}	99	59	66	35	99	64	42	31	100	32	42	100
10-C side chain	77	32	48	60	72	46	23	49	78	30	38	85
9-C side chain	22	65	51	39	28	53	71	50	22	64	61	15

[a]Values given are the percentage of total sterols with a given feature. Totals exceed 100% because more than 1 listed feature may be present in a given molecule.

plant cells, grown in suspension culture, appear to be as sensitive to the fungicide as fungi previously studied. However, an intact higher plant may be more resistant to triarimol. When bean seedlings were treated with the fungicide, no noticeable modification in the sterol content was observed (7).

Effects of Tridemorph on Sterol Composition

The sterol compositions of carrot, tobacco and soybean cultures treated with tridemorph differed from the sterol composition of control cultures. The occurrence of 24-methylenepollinastanol (14α-methyl-9β,19-cyclo-5α-ergost-24(28)-en-3β-ol) was detected in carrot and tobacco (Table 2), but not in soybean cultures treated with tridemorph. The molecular ion of the sterol was at m/z 412 (5%). The spectrum was similar to the one described by Doyle et al. (22). 24-Methylenepollinastanol is also one of the major cyclopropyl sterols to accumulate in suspension culture of bramble (11) and in corn seedlings (12) treated with tridemorph.

In tridemorph-treated cultures of tobacco and soybean, 24-methylpollinastanol (14α-methyl-9β,19-cyclo-5α-ergostan-3β-ol) was detected, but was not observed in carrot cultures (Table 2). The molecular ion was at m/z 414 (22%). The prominent ions were at m/z 399 ($M-CH_3$, 36%), 396 ($M-H_2O$, 35%), 381 ($M-CH_3-H_2O$, 29%), 302 (cleavage of cyclopropane ring and ring B, 42%) and 287 (M-side chain, 45%). This cyclopropyl sterol accumulated in cultures of bramble (11) and in corn seedlings (12) treated with the fungicide. 28-Isofucosterol was detected in carrot and tobacco cultures but not in soybean (Table 2) cultures. The MS was similar to the spectrum of authentic samples. This sterol was present in the untreated cultures of bramble, but not detected when treated with tridemorph (11). In contrast, 28-isofucosterol was not detected in any of the control cultures of carrot, tobacco or soybean used in this study.

24-Methylenecholesterol (ergosta-5,24(28)-dien-3β-ol) accumulation was limited to tobacco cultures (Table 2) treated with the fungicide. The occurrence of 24-methylene cholesterol was noted in untreated cultures of bramble cells (11). In our study, 24-methylenecholesterol was not detected in the control cultures of carrot, tobacco or soybean.

The MS of remaining sterols from tridemorph-treated cultures, cholesterol, campesterol, 14α-methyl-5α-ergost-8-en-3β-ol and 24-methylenecycloartanol were identical to the MS of the same sterols found in either control or triarimol-treated cultures.

In bramble cells (11) and corn seedlings

(12), tridemorph is known to inhibit the enzyme involved in the opening of cyclopropane ring. In our study, the fungicide caused a similar effect in carrot, tobacco and soybean cultures. A tremendous accumulation of sterols with a cyclopropane ring occurred in tridemorph-treated cultures. Ca. 33% of total sterol in carrot, 58% in tobacco and 49% in soybean (Table 3) cultures treated with tridemorph were sterols with a cyclopropane ring. An increase in the concentration of cyclopropyl sterols resulted from the accumulation of cycloeucalenol, 24-methylenecycloartanol, 24-methylenepollinastanol and 24-methylpollinastanol in tridemorph-treated cultures (Table 2).

An enzyme in cell-free preparations of bramble cells (36) and in microsomes of corn embryo (37,38) was shown to cleave the cyclopropane ring of cycloeucalenol and isomerize the sterol to obtusifoliol. This enzyme, cycloeucalenol-obtusifoliol isomerase from corn embryo, was shown to be highly specific for cycloeucalenol (37,38). In tridemorph-treated cultures of carrot, tobacco and soybean, cyclopropyl sterols accumulated, but obtusifoliol was not detected in any of the fungicide-treated cultures (Table 2). The absence of obtusifoliol and the presence of cyclopropyl sterols, e.g., cycloeucalenol, in the treated cultures indicate that the fungicide blocks the conversion of cycloeucalenol to obtusifoliol. Tridemorph appears to be an effective inhibitor of the cycloeucalenol-obtusifoliol isomerase in carrot, tobacco and soybean cells.

The other major tridemorph-induced modification related to the sterol composition was that tridemorph caused a reduction of sterols with a 10-carbon side chain and an increase of sterols with a 9-carbon side chain (Table 3). In addition, the level of 24-methylene sterols increased in the fungicide-treated cultures, especially in carrot and tobacco (Table 3) cultures. In view of the effects in these cultures, tridemorph appears to be an effective inhibitor of the second alkylation at carbon 24.

A minor effect of tridemorph may be to prevent the removal of the 14α-methyl group in soybean cultures, where 8% of total sterol was 14α-methyl-5α-ergost-8-en-3β-ol (Table 2). The other secondary effect of the fungicide was on the accumulation of sterols with $\Delta^{5,24(28)}$ double bonds, e.g., 24-methylenecholesterol and 28-isofucosterol (Table 2). Because these sterols were not detected in controls, the presence of $\Delta^{5,24(28)}$ sterols in treated cultures (Table 3) indicates that tridemorph inhibits the reduction of the $\Delta^{24(28)}$-double bond.

Tridemorph has been known to inhibit the $\Delta^8 \rightarrow \Delta^7$ isomerase in fungi (2,3,10). Previously the inhibition of this enzyme was demonstrated for corn seedlings treated with low concentrations of the fungicide (12). This effect of tridemorph was observed here only in cultures of soybean with the accumulation of 14α-methyl-5α-ergost-8-en-3β-ol. Instead the primary effect of tridemorph was the inhibition of the opening of the 9β,19-cyclopropane ring.

When relative sensitivities of tridemorph are compared in fungi and higher plants, a trend similar to that observed with triarimol is found.

In fungi tested (2,3,9,10), the concentration of tridemorph effective in inducing the primary effect (inhibition of the $\Delta^8 \rightarrow \Delta^7$ isomerase) was in the range of 3-10 mg/l. In higher plants, the primary effect of tridemorph appears to be the accumulation of 9β,19-cyclopropyl sterols. In carrot, tobacco and soybean suspension cultures, the concentrations of tridemorph effective in the accumulation of cyclopropyl sterols were 3 mg/l, 1 mg/l and 5 mg/l, respectively. In suspension cultures of bramble, the concentration of tridemorph effective in the accumulation of cyclopropyl sterols was 10 mg/l (11). An intact higher plant, however, was less sensitive to the fungicide. In corn seedlings, the accumulation of 9β,19-cyclopropyl sterols resulted when treated with 20 mg/l of tridemorph (12).

Effects of Triparanol on Sterol Composition

Sterol compositions of carrot and tobacco cultures were altered when treated with triparanol (Table 2). However at 16 mg/l, triparanol did not greatly change the sterol composition of soybean cultures (Table 2). All sterols detected in triparanol-treated cultures were observed in either triarimol or tridemorph-treated cultures. The structural features of sterols from triparanol-treated cultures were identical to the sterols from triarimol- or tridemorph-treated cultures.

The effects of triparanol on sterol composition were quite similar in carrot and in tobacco cultures. In carrot and tobacco cultures treated with triparanol, the accumulation of sterols with a 14α-methyl group was significant (Table 3). In particular, 14α-methyl-5α-stigmast-8-3n-3β-ol and 14α-methyl-5α-ergost-8-en-3β-ol were major 14α-methyl sterols accumulating in these cultures. The accumulation of these 14α-methyl sterols contributed to the major build-up of $\Delta^{8(9)}$-sterols with a 14α-methyl group (Table 3). The accumulation of sterols with a 14α-methyl group and the absence of sterols with a 4α-methyl group indicate that triparanol is an effective inhibitor of the 14α-demethylase in carrot and tobacco cultures. A similar effect when a 14α-methyl group was

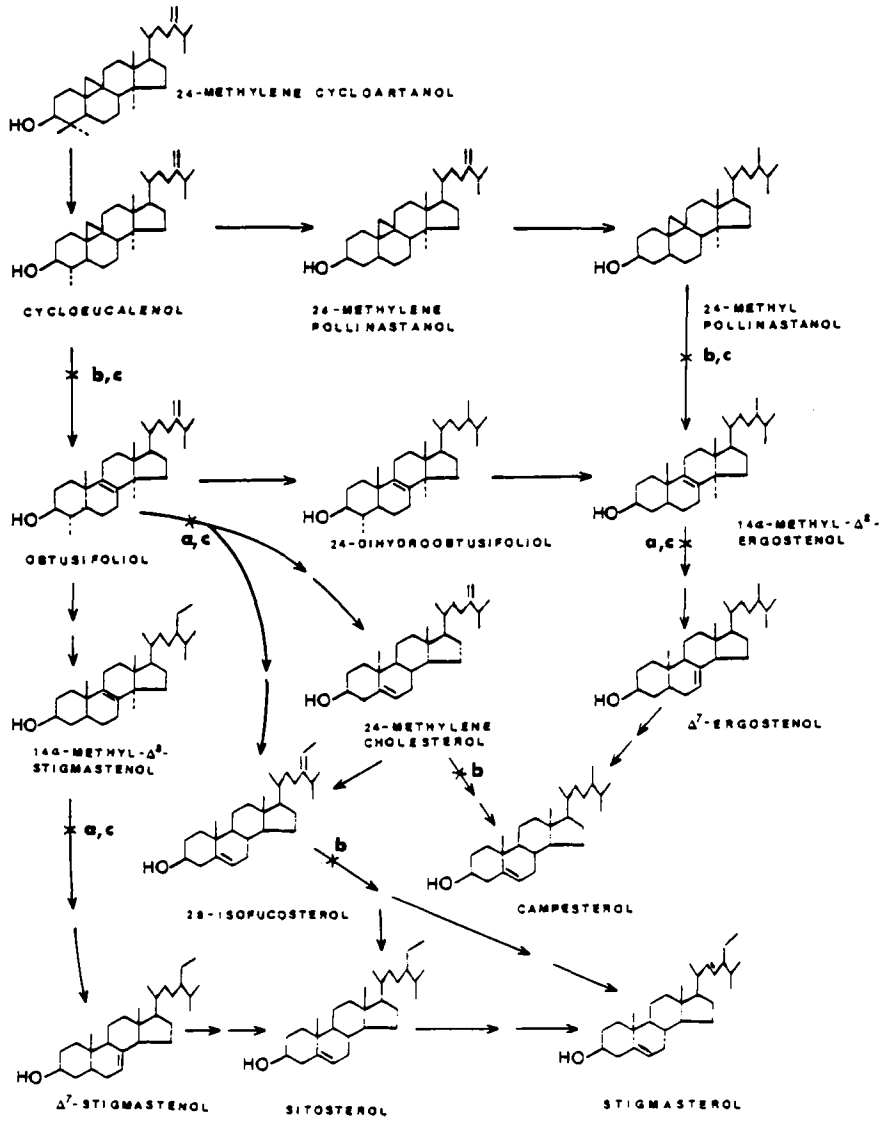

FIG. 1. Sites of inhibition of (a) triarimol, (b) tridemorph and (c) triparanol in plant tissue cultures.

removed was observed in *Chlorella* species treated with triparanol (19-22).

Just as the accumulation of sterols with 9β,19-cyclopropane ring was noted in *C. emersoni* treated with triparanol (19,22), the accumulation of cyclopropyl sterols also occurred in carrot and tobacco cultures treated with triparanol (Table 3). In triparanol-treated cultures of carrot, the major cyclopropyl sterols accumulated were 24-methylenecycloartanol, cycloeucalenol, 24-methylenepollinastanol and 24-methylpollinstanol (Table 2).

In the case of tobacco cultures, cyclopropyl sterols accumulated and larger accumulations of sterols occurred with a 14α-methyl group (in particular obtusifoliol) when treated with triparanol (Table 3).

When soybean cells were treated with triparanol, only the normally occurring Δ^5-sterols campesterol, stigmasterol and sitosterol were present. However, campesterol decreased from 22% of total sterol in the control to 16% in the treated cultures, stigmasterol decreased from 37% to 26%, but sitosterol increased from 41% to 59% in triparanol-treated cultures of soybean (Table 2). Triparanol has been known to inhibit the synthesis of Δ^{22}-sterols in *Chlorella* species (19).

The sites of sterol biosynthesis inhibition by triarimol, tridemorph and triparanol demonstrated in this work are illustrated in Figure 1.

REFERENCES

1. Mitropoulos, K.A., Gibbons, G.L., Connell, C.M., and Woods, R.A. (1976) Biochem. Biophys. Res. Commun. 71, 892-900.
2. Berg, L.R. (1983) The Effects of Triarimol and Tridemorph on the Sterol Biosynthesis of Five Species of Oomycetes, Ph.D. Thesis, University of Maryland, College Park, MD, pp. 1-43.
3. Berg, L.R., Patterson, G.W., and Lusby, W.R. (1983) Lipids 18, 448-452.
4. Ragsdale, N.N. (1975) Biochem. Biophys. Acta 380, 81-96.
5. Ragsdale, N.N., and Sisler, H.D. (1972) Biochem. Biophys. Res. Commun. 46, 2048-2053.
6. Frasinel, C., Patterson, G.W., and Dutky, S.R. (1978) Phytochemistry 17, 1567-1570.
7. Shive, J.B., and Sisler, H.D. (1976) Plant Physiol. 57, 640-644.
8. Schmitt, P., and Benveniste, P. (1979) Phytochemistry 18, 1659-1665.
9. Kerkenaar, A., Barug, D., and Sijpesteijn, A.K. (1979) Biochem. Physiol. 12, 195-204.
10. Kato, T., Shoami, M., and Kawase, Y. (1980) J. Pesticide Sci. 5, 69-79.
11. Schmitt, P., Benveniste, P., and Leroux, P. (1981) Phytochemistry 20, 2153-2159.
12. Bladocha, M., and Benveniste, P. (1983) Plant Physiol. 71, 756-762.
13. Avigan, J.D., Steinberg, D., Vroman, H.E., Thompson, M.J., and Mosettig, E. (1960) J. Biol. Chem. 235, 3123-3126.
14. Steinberg, D., and Avigan, J. (1960) J. Biol. Chem. 235, 3127-3129.
15. Svoboda, J.A., and Robbins, W.E. (1967) Science 156, 1637-1638.
16. Cole, R.L.J., and Krusberg, L.R. (1968) Life Sci. 7, 713-724.
17. Fung, B., and Holmlund, C.E. (1976) Biochem. Pharmacol. 25, 1249-1254.
18. Campagnoni, C., Holmlund, C.E., and Whittaker, N. (1977) Arch. Biochem. Biophys. 184, 555-560.
19. Patterson, G.W., Doyle, P.J., Dickson, L.G., and Chan, J.T. (1974) Lipids 9, 567-574.
20. Chan, J.T., and Patterson, G.W. (1973) Plant Physiol. 52, 246-247.
21. Chan, J.T., Patterson, G.W., Dutky, S.R., and Cohen, C.F. (1974) Plant Physiol. 53, 244-249.
22. Doyle, P.J., Patterson, G.W., Dutky, S.R., and Thompson, M.J. (1972) Phytochemistry 11, 1951-1960.
23. Murashige, T., and Skoog, F. (1962) Physiol. Plantarum 15, 473-497.
24. Linsmaier, E.M., and Skoog, F. (1965) Physiol. Plantarum 18, 100-127.
25. Tu, C., Powrie, W.D., and Fennema, O. (1968) Lipids 4, 369-379.
26. Vroman, H.E., and Cohen, C.F. (1967) J. Lipid Res. 8, 150-152.
27. Patterson, G.W. (1971) Anal. Chem. 43, 1165-1170.
28. Knights, B.A. (1967) J. Gas Chromatogr. 5, 273-282.
29. Benveniste, P., Hirth, L., and Ourisson, G. (1966) Phytochemistry 5, 31-44.
30. Song, M., and Tattrie, N. (1973) Can. J. Bot. 51, 1893-1897.
31. Radwin, S.S., Spener, F., Mangold, H.K., and Staba, E.J. (1975) Chem. Phys. Lipids 14, 72-80.
32. Williams, B.L., and Goodwin, T.W. (1965) Phytochemistry 4, 81-88.
33. Heble, M.R., Narayanaswami, S., and Chadha, M.S. (1968) Science 145, 1145.
34. Doyle, P.J., Patterson, G.W., Dutky, S.R., and Cohen, C.I. (1971) Phytochemistry 10, 2093-2098.
35. Goodwin, T.W. (1979) Ann. Rev. Plant Physiol. 30, 369-404.
36. Heintz, R., and Benveniste, P. (1974) J. Biol. Chem. 249, 4267-4274.
37. Rahier, A., Cattel, L., and Benveniste, P. (1977) Phytochemistry 16, 1187-1192.
38. Rahier, A., Benveniste, P., and Cattel, L. (1976) J. Chem. Soc. Chem. Commun., 287-288.

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Free and Esterified Sterols of the Marine Dinoflagellate *Gonyaulax polygramma*

JOHN K. VOLKMAN,[a]* ROBERT B. GAGOSIAN[b] and STUART G. WAKEHAM,[b]
[a]CSIRO Division of Oceanography, G.P.O. Box 1538, Hobart, Tasmania, 7001, Australia; and [b]Department of Chemistry, Woods Hole Oceanographic Institution, Woods Hole, MA 02543

ABSTRACT

Free and esterified sterols of the common marine dinoflagellate *Gonyaulax polygramma* were identified using capillary gas chromatography–mass spectrometry (GC-MS). Fractions containing free 4α-methyl and 4-desmethyl sterols were isolated by column chromatography and shown to consist of at least 20 components. Major sterols included 4α,23,24-trimethyl-5α-cholestan-3β-ol (dinostanol), 4α,23,24-trimethyl-5α-cholest-22E-en-3β-ol (dinosterol), cholest-5-en-3β-ol (cholesterol), 23,24-dimethyl-5α-cholest-22E-en-3β-ol and 23,24-dimethylcholesta-5,22E-dien-3β-ol. Although the same group of sterols was found in the free and esterified sterol fractions, the proportions of individual sterols were quite different. The complexity of the sterol distributions, together with the predominance of dinostanol, distinguishes the sterol composition of this alga from those of other members of the *Gonyaulacaceae*.
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INTRODUCTION

Dinoflagellates are often major constituents of marine phytoplankton and thus are important in many marine food chains. Recent biochemical studies have focussed on the identification of toxins produced by species that form massive blooms called red tides (1). Considerable interest has also been shown in the sterol composition of dinoflagellates, both as a source of novel sterol structures (2-19), and in studies of the cellular interactions between marine animals and their symbiotic zooxanthellae (20-24).

Dinoflagellates are also a major source of the organic matter found in some marine sediments, and hence they can be significant contributors of the sterols and other lipids found in these sediments (26,27). For example, the presence of dinosterol, 4α,23,24-trimethyl-5α-cholest-22E-en-3β-ol, in marine sediments is taken as good evidence that at least part of the organic matter originated from dinoflagellates (26,27) because this unusual sterol is common in these algae (2-4,12,13) but is not found in others.

Unfortunately most reports of dinoflagellate sterols in the literature do not record all the minor sterols present, mainly because the analyses were performed on low resolution packed gas chromatography (GC) columns and not on capillary columns that are widely used today. Such data are essential for organic geochemical studies that attempt to assign origins to the sedimentary organic matter. In fact, many sterols of presumed dinoflagellate origin that have been found in sediments (27) have yet to be identified in these algae. With this in mind, we undertook a detailed analysis of a common red-tide marine dinoflagellate, *Gonyaulax polygramma* (25), using capillary GC and gas chromatography-mass spectrometry (GC-MS) techniques comparable to those used in organic geochemical studies.

Our analyses show that *Gonyaulax polygramma* contains a much more diverse range of sterols than had previously been reported for other *Gonyaulax* species (2,4,5,7), which were based on packed column analyses. Further, the major sterol is not dinosterol but its fully saturated analog 4α,23,24-trimethyl-5α-cholestan-3β-ol. The biosynthetic implications of these results are discussed.

EXPERIMENTAL

Gonyaulax polygramma Stein was cultured by S. Gallagher at Environmental Systems Laboratory, Woods Hole Oceanographic Institution, using f/2 culture medium (28) bubbled with air at 10 ml/min. The alga was cultured in 5 l and 10 l batches for 14 days at 18 ± 1 C under a white light intensity of 5×10^{-4} watts/cm^2. Cells were collected during the exponential phase of growth, washed briefly with distilled water and extracted with CH_2Cl_2 using

*To whom correspondence should be addressed.

sonication to disrupt the cells. The total nonpolar lipid extract was separated into lipid classes by column chromatography using a 0.9 cm i.d. glass column packed to a depth of 22 cm with silicic acid (70-230 mesh), deactivated with 5%, by weight, of water. Fourteen fractions were collected by elution with hexane, toluene and ethyl acetate mixtures. After elution with hexane (50 ml), and 25% toluene in hexane (20 ml), a fraction containing steryl esters was eluted with 50% toluene in hexane (10 ml). After further elution with 50% toluene in hexane (10 ml), 5% ethyl acetate in hexane (20 ml) and 10% ehtyl acetate in hexane (20 ml), a fraction containing 4α-methyl sterols was eluted with 15% ethyl acetate in hexane (20 ml). Further elution with 20% ethyl acetate in hexane (20 ml) afforded a fraction containing 4-desmethyl sterols. No desmethyl sterols or 4-methyl sterols were detected in the column fractions eluting before or after the sterol fractions. The free sterols were reacted with acetic anhydride in pyridine to convert the sterols to steryl acetates for GC and GC-MS analysis. An aliquot of the steryl ester fraction was hydrolyzed in methanolic KOH, and the total sterols liberated were converted to steryl acetates, without further separation.

Each sterol fraction was analyzed by capillary GC using a Carlo Erba FTV 2150 gas chromatograph fitted with a 20 m × 0.33 mm i.d. pyrex WCOT column coated with SE-52. The samples were injected in the splitless mode at room temperature and the oven was then programmed to 330 C at 4 C/min. Each fraction was also analyzed on a 25 m × 0.2 mm i.d. OV-1 fused-silica capillary column using a Shimadzu GC-9A gas chromatograph with H_2 as the carrier gas. The oven was operated isothermally at 260 C to enable retention indices relative to cholesteryl acetate to be calculated for comparison with literature data (27,29,41). Compounds were identified by coinjection with standards, comparison of retention index data and from MS data. Sterol abundances were calculated from flame ionization detector (FID) responses assuming equal response factors. Intact steryl esters were also analyzed directly by GC using persilylated SE-52 glass capillary columns programmed from 180 C to 370 C at 2 C/min (30). A nonvaporizing, cold on-column injector was used for these analyses to minimize discrimination against high molecular weight components (30). Reference standards were not available for most of the steryl esters present so no attempt was made to identify individual compounds. Retention times indicate that the fatty acids contain C14 to C20 chain lengths, with palmitic acid as a major component.

Electron impact MS of steryl acetates were obtained using a Finnigan 1015C quadrupole mass spectrometer coupled to a Varian Aerograph 1400 gas chromatograph modified for capillary column operation (30). A 25 m × 0.3 mm i.d. SE-52 WCOT pyrex capillary column was used with He at 55 kPa as the carrier gas, and GC conditions as above. The mass spectrometer was scanned from 50 a.m.u. to 550 a.m.u. in 1 sec intervals. A Finnigan-INCOS 2300 data system was used to acquire and process the MS data. Portions of the reconstructed ion chromatograms of the free 4α-methyl sterol and 4-desmethyl sterol fractions (as acetates) are shown in Figure 1.

RESULTS

Compositional data for free and esterified 4-desmethyl sterol and 4α-methyl sterol fractions are presented in Table 1. From the amount of material in the 2 fractions, the ratio of free 4α-methyl sterols to free 4-desmethyl sterols was calculated to be ca. 1.1:1. The corresponding ratio for the esterified sterols was 3.5:1. The ratio of free-to-esterified sterols was ca. 11:1.

Free 4-Desmethyl Sterols

This fraction consisted of at least 14 components, of which 8 were positively identified and a further 5 tentatively identified. Sterols 1-6 (Fig. 1A) were identified as cholesta-5,22E-dien-3β-ol, cholest-5-en-3β-ol, 5α-cholestan-3β-ol, 24-methylcholesta-5,22E-dien-3β-ol, 24-methylcholest-5-en-3β-ol and 24-methyl-5α-cholestan-3β-ol from coinjection in each case with the appropriate standard and by comparison of MS with published data (29). The possibility that sterol 1 might have the structure 27-nor-24-methylcholesta-5,22E-dien-3β-ol (occelasterol) was considered because this sterol has a very similar MS to cholesta-5,22E-dien-3β-ol. However, these 2 sterols are completely separated by apolar capillary columns (e.g., 27) and coinjection with standards confirmed that sterol 1 was cholesta-5,22E-dien-3β-ol. Configurations at C24 could not be determined because of the small amounts of sterols isolated.

Sterol 7 is a diunsaturated C29 sterol, identified as 23,24-dimethylcholesta-5,22E-dien-3β-ol from its retention index (1.34 cf. 1.36 [41]) and MS (MS m/z [relative intensity] 394[M-60, 7%], 379[0.1], 351[1], 323[0.4], 282[1.7], 267[0.6], 255[5], 253[5], 69[100]). The MS of an alternative structure, 24-ethylcholesta-5,22E-dien-3β-ol, is quite different (29), and does not have a major ion at m/z 69, which is

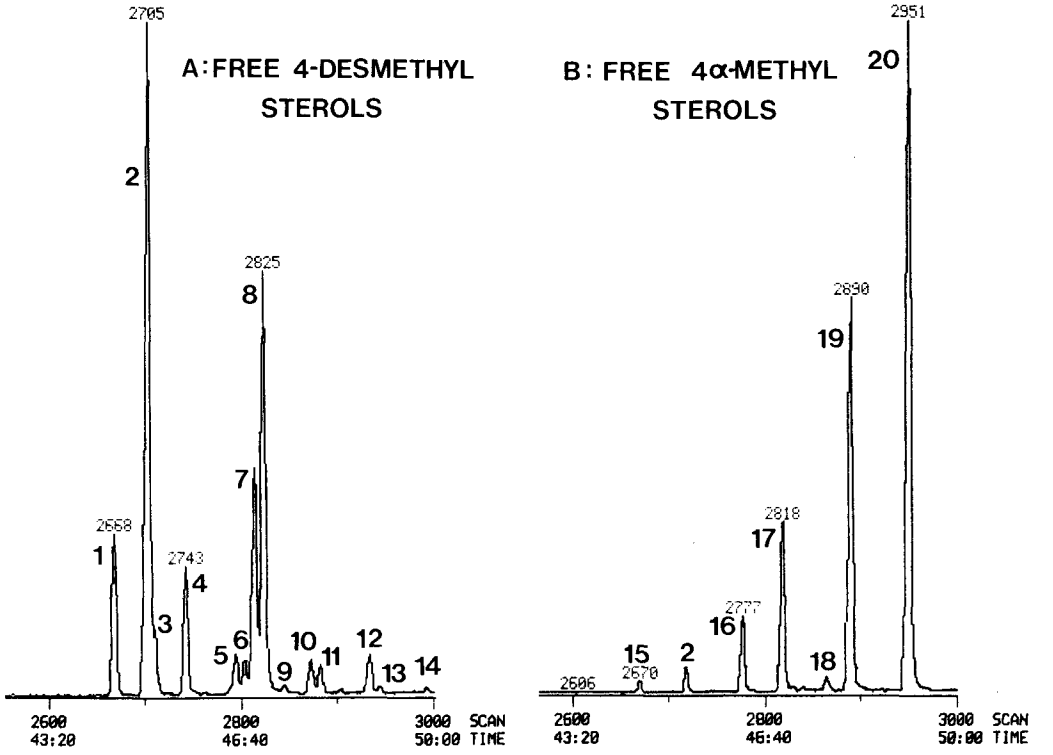


FIG. 1. Partial reconstructed total ion chromatograms (RIC) of A: 4-desmethyl sterols (as acetates) and B: 4α-methyl sterols (as acetates). Compounds are identified in Table 1. SE-52 capillary column, conditions as described in the Experimental section.

characteristic of sterols with Δ22-23,24-dimethyl side chains. This sterol also elutes slightly later on apolar capillary columns (31). The MS of sterol 8 indicated a sterol with very similar structure to sterol 7 but with one less double bond (MS: 456[M^+,0.3%], 413[0.4], 353[1], 344[3], 315[6], 257[7], 69[100]). This compound was identified as 23,24-dimethyl-5α-cholest-22E-en-3β-ol because it elutes just after 23,24-dimethylcholesta-5,22E-dien-3β-ol with a retention index difference of 0.04, which is typical of Δ5,22-sterol/Δ22-sterol pairs (41). Also, the presence in the MS of the ion m/z 257 is indicative of a saturated nucleus and hence a side-chain double bond is present in this compound.

Sterols 10 (MS: 396[M-60$^+$,35%], 381[2], 255[3], 213[6], 147[100]) and 11 (MS: 458[M^+,0.2%], 398[0.6], 257[0.3], 215[31], 98[100]) were identified as 23,24-dimethyl-cholest-5-en-3β-ol and 23,24-dimethyl-5α-cholestan-3β-ol from their MS and retention indices (27), but these identifications could not be confirmed because authentic standards were not available. The MS of compound 12 (MS: 382 [M^+,26], 367[2], 187[21], 174[100], 161[35]) was very similar to a library spectrum of cholesta-3,5-dien-7-one but a standard was not available for coinjection. From its retention time, this compound seems more likely to be cholest-5-en-3β-ol-7-one, in which case the m/z 382 ion corresponds to loss of acetic acid from the acetate derivative.

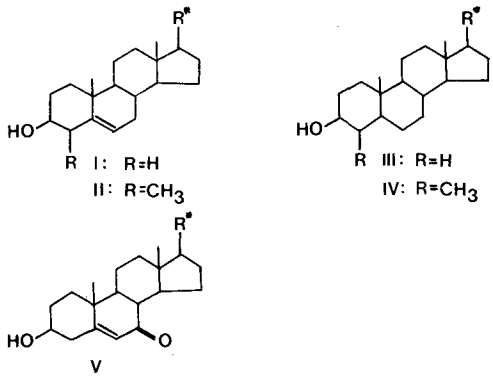


FIG. 2. Tetracyclic ring systems occurring in sterols of *G. polygramma*. Side chains (R*) are given in Schemes 1 and 2.

TABLE 1

Sterol Composition of *Gonyaulax polygramma*

GC-MS Peak[a]	RRT[b] This work	RRT[b] Ref. 41	Sterol	Structure number[c]	Carbon number[d]	Weight (%) Free	Weight (%) Esterified
			4-Desmethyl sterols				
1	0.92	0.92	Cholesta-5,22E-dien-3β-ol	Ig	27:2	8.9	8.7
2	1.00	1.00	Cholest-5-en-3β-ol	Ik	27:1	36.2	54.2
3	1.03	1.03	5α-Cholestan-3β-ol	IIIk	27:0	2.5	2.7
4	1.11	1.12	24-Methylcholesta-5,22E-dien-3β-ol	Id	28:2	7.1	3.8
5	1.28	1.29	24-Methylcholest-5-en-3β-ol	Ic	28:1	1.5	tr
6	1.31	1.33	24-Methyl-5α-cholestan-3β-ol	IIIc	28:0	1.5	tr
7	1.34	1.36	23,24-Dimethylcholesta-5,22E-dien-3β-ol	Ie	29.2	12.2	1.9
8	1.38	–	23,24-Dimethyl-5α-cholest-22E-en-3β-ol	IIIe	29:1	22.7	24.7
10	1.55	–	23,24-Dimethylcholest-5-en-3β-ol	If	29:1	1.9	tr[f]
11	1.60	–	23,24-Dimethyl-5α-cholestan-3β-ol	IIIf	29:0	1.7	tr
12	1.74	–	Cholesta-5-en-3β-ol-7-one?	Vk	27:1	2.1	–
			Others[e] (each < 1.2%)			1.7	4.0
						100.0	100.0
			4α-Methyl sterols				
16	1.18	1.19	4α-Methyl-5α-cholestan-3β-ol	IVk	28:0	5.8	6.0
17	1.30	–	4α,24-Dimethyl-5α-cholest-22E-en-3β-ol	IVd	29:1	12.7	31.0
18	1.45	–	4α,24-Dimethyl-5α-cholest-24(28)-en-3β-ol	IVb	29:1	1.5	2.9
–	1.48	–	4α,24-Dimethyl-5α-cholestan-3β-ol	IVc	29:0	tr	2.9
19	1.56	–	4α,23,24-Trimethyl-5α-cholest-22E-en-3β-ol	IVe	30:1	29.4	24.2
20	1.83	–	4α,23,24-Trimethyl-5α-cholestan-3β-ol	IVf	30:0	50.1	31.1
			Others (each < 0.5%)			0.5	1.9
						100.0	100.0

[a]Refers to Figure 1.
[b]Retention times relative to cholesteryl acetate=1.00 (OV 1 capillary column).
[c]Side chains a-j are given in Schemes 1 and 2; tetracylic ring systems I-V in Fig. 2. Side chain k is side chain a without the double bond.
[d]Number of carbon atoms/number of double bonds.
[e]Trace amounts of 24-methylcholesta-5,24(28)-dien-3β-ol (Ib) and 24-ethylcholesta-5,24(28)Z-dien-3β-ol were also identified (see results section).
[f]Tr: <1%.

Sensitive GC-MS techniques were used to tentatively identify several minor sterols. A mass fragmentogram for the ion m/z 296, which is a characteristic feature of the MS of steryl acetates containing a 24(28) double bond (e.g., 29), indicated the presence of 2 minor sterols with this structural feature. The first compound corresponded with the small peak on the upslope of peak 5 in Figure 1, which we confirmed was the expected position for 24-methylcholesta-5,24(28)-dien-3β-ol by coinjection with an authentic standard. The calculated retention index of 1.28 on SE-52 also compares well with the literature value of 1.27 for OV 1 columns (41). Djerassi (40) has drawn attention to the fact that another sterol, 22,23-methylenecholesterol, could be mistakenly identified as 24-methylcholesta-5,24(28)-dien-3β-ol, because these sterols have similar MS, but they quote a retention index of 1.22 on SE-52, which is clearly different from that of the minor sterol in *Gonyaulax polygramma*. The m/z 296 mass fragmentogram also revealed the presence of a second sterol containing a 24(28) double bond eluting between sterols 11 and 12 at scan number 2904 (Fig. 1). This compound has a retention index of 1.67, which is identical with that reported by Itoh et al. (41) for 24-ethylcholesta-5,24(28)Z-dien-3β-ol (28-isofucosterol). No peak was found at the expected retention time for the 24(28)E double-bond isomer, fucosterol. 28-Isofucosterol has also been identified in *Gonyaulax diagenesis* (5).

Free 4α-Methyl Sterols

This fraction consisted of 4 major and 2 minor constituents, plus a trace amount of cholesterol that was incompletely separated during column chromatography. Compound 16 was identified as 4α-methyl-5α-cholestan-3β-ol from its characteristic MS and retention index (MS: 444[M^+,0.5%], 384[16], 369[20], 355 [3], 244[9], 229[79], 215[15], 95[100]). The MS of sterol 17 indicated a monounsaturated C29 sterol (MS: 456[M^+,4%], 358[6], 329[7], 271[13], 95[100]); ions at m/z 271 and 358 indicated that the double bond is in the side chain, probably at C22. This sterol coeluted with an authentic standard of 4α,24-dimethyl-5α-cholest-22E-en-3β-ol, indicating that it is probably not the structurally similar sterol 4α,23-dimethyl-5α-cholest-22E-en-3β-ol, which is a major constituent of *Gonyaulax diagenesis* (5). Coinjection with a standard also confirmed the presence of minor amounts of the sterol, 4α,24-dimethyl-5α-cholest-24(28)-en-3β-ol (sterol 18), which was confirmed from its MS (MS: 372[M^+-84,100], 357[9], 329[28], 269[13], 229[29], 95[67]).

Sterol 19 was identified as dinosterol (4α,-23,24-trimethyl-5α-cholest-22E-en-3β-ol) from its MS (12) and by coinjection (MS: 470[M^+, 0.1%], 398[0.2], 367[0.8], 358[2.8], 329[7], 271[7], 229[0.4], 69[100]). The MS of the major constituent of the 4α-methyl sterol fraction, compound 20, indicated that it is a saturated C30 sterol (m/z 472) with a C10 side chain (MS: 472[M^+,1.3%], 412[4.6], 397[5.5], 383[1.2], 229[21], 98[100]). This sterol was identified as dinostanol (4α,23,24-trimethyl-5α-cholestan-3β-ol) by coinjection with the stanol produced from catalytic hydrogenation of dinosterol. An alternative possibility for sterol 20, 4α-methyl-24-ethyl-5α-cholestan-3β-ol, was ruled out because on the OV 1 capillary column used in our work it eluted slightly before dinostanol, although on packed columns it would probably coelute. The MS of these 2 steryl acetates are very similar but the MS of 4α-methyl-24-ethyl-5α-cholestan-3β-yl acetate (MS: 472[M^+,6.3%], 412[20], 397[34], 383[7.1], 229[75], 95[100]) lacks a major ion at m/z 98.

Esterified Sterols

Saponification of the steryl ester fraction yielded a mixture containing 4α-methyl and 4-desmethyl sterols that was acetylated and analyzed without further separation. The fatty acid moieties were not examined. Almost all of the sterols found in the free fraction were also identified in the steryl ester fraction but quantitative differences were apparent in the 2 distributions (Table 1). The esterified 4-desmethyl sterol fraction contained considerably more cholesterol than the free sterols (54.2 cf. 36.2%), and much less 23,24-dimethylcholesta-5,22E-dien-3β-ol (1.9 cf. 12.2%). Dinostanol predominated in the esterified 4α-methyl sterols as in the free sterols, although its relative abundance was diminished (31.1 cf. 50.1%). However, the second major esterified 4α-methyl sterol was 4,24-dimethyl-5α-cholest-22-en-3β-ol (31.0%) and not dinosterol as in the free 4α-methyl sterols. Several minor sterols were detected in the saponification products of the steryl ester fraction that were not present in the free sterols but their concentrations were too low to permit unequivocal identifications.

DISCUSSION

The sterol composition of *Gonyaulax polygramma* differs from that of other *Gonyaulax* species in several major respects. First, many *Gonyaulax* species contain cholesterol and

dinosterol with only small amounts of other sterols (2,4), but in *G. polygramma*, the combined abundance of these 2 sterols represents only 34% of the total free sterols (Table 1). Second, the wide range of sterol structures biosynthesized by *G. polygramma* is unusual and found in only a few other algae. Third, the high proportion of the 2 C29 4-desmethyl sterols, 7 and 8, in the 4-desmethyl sterol fraction is without precedent, as is the predominance of dinostanol in the 4α-methyl sterol fraction.

4-Desmethyl Sterols

Cholesterol has been identified as the sole or major 4-desmethyl sterol in most *Gonyaulax* species (4) analyzed to date. Isofucosterol has been reported, however, in *G. diagenesis* (5), and gorgosterol and 23-methylene-24-methylcholestanol have been reported in *G. monilata* (7). Despite the diversity of 4-desmethyl sterols synthesized by *G. polygramma*, only a trace of 1 of these 3 sterols (isofucosterol) was detected in our analysis. Most of the 4-desmethyl sterols identified are present, however, in other species of dinoflagellates.

Cholesta-5,22E-dien-3β-ol is found in a variety of organisms in the marine environment, including the luminescent dinoflagellate *Pyrocystis lunula* (9), and several species of zooxanthellae from corals (21) and a zoanthid (10), although it is rarely a major component (32). 24-Methylcholesta-5,22E-dien-3β-ol is common in diatoms (32,33), but it is comparatively rare in dinoflagellates with the exception of the dinoflagellate *Noctiluca milialis*, where it represents over 80% of the free and esterified sterols (19). It has been found as a minor sterol in several zooxanthellae (21).

The identification of small amounts of 4-desmethyl stanols, e.g., 5α-cholestan-3β-ol, is of interest because saturated sterols are very uncommon in marine unicellular algae (18). 5α-Cholestan-3β-ol has been detected in the dinoflagellates *Pyrosystis lunula* (9,34) and *Noctiluca milialis* (19) and it is a minor constituent of the cultured zooxanthellae isolated from a coral (17). Small amounts of ring-saturated 4-desmethyl sterols have also been detected in an alga FCRG51 assigned to the Dinophyceae on chemotaxonomic grounds (18). Significant amounts (17.2% of total sterols) also occur in the luminescent dinoflagellate *Pyrocystis lunula* (34). Ring-saturated sterols represent only 27% of the free 4-desmethyl sterols, but they represent at least 99% of the free 4α-methyl sterols. The corresponding values for the esterified sterol fraction are 25.5% and 98%, respectively.

23,24-Dimethylcholesta-5,22E-dien-3β-ol is widely distributed in the marine environment, being found in molluscs (35), soft corals (36), a diatom (32) and a coccolithophore (33), but it has been isolated previously from only one cultured dinoflagellate (34). It has also been found in an alga, FCRG51, (18) and in some coral zooxanthellae that are probably dinoflagellates (24). Only 3 other reports (17,18,34) have been made of the isolation of 23,24-dimethyl-5α-cholest-22E-en-3β-ol from cultured algae, despite the fact that this sterol is the 4-desmethyl analog of dinosterol, which is the major sterol in most dinoflagellates. This sterol is also thought to be a biosynthetic intermediate in the biosynthesis of gorgosterol (37) but no trace of the latter could be detected in *G. polygramma*. No reports seem to exist of the more saturated 23,24-dimethyl sterols 10 and 11 in marine biota, although both have been identified in marine sediments (27). Our data support the suggestion of De Leeuw et al. (27) that in sediments these sterols may originate from dinoflagellates.

4α-Methyl Sterols

The high concentration of dinosterol in *G. polygramma* is typical of most dinoflagellates, but one other report has been made of its fully saturated analog, dinostanol, in a dinoflagellate (8) and it was only a minor constituent in that alga. Several reports have been made, however, of the structurally related stanol, 4α-methyl-24-ethyl-5α-cholestan-3β-ol, in cultured dinoflagellates (11), and zooxanthellae (17,22). We have shown that these 2 stanols have very similar retention times (see Results section), even on high resolution capillary columns such as used here, so that MS data are essential to differentiate between them if packed columns are used.

Of the other two 4α-methyl sterols in *G. polygramma*, 4α-methyl-5α-cholestan-3β-ol has been identified as a very minor constituent of *Noctiluca milialis* (19), *Pyrocystis lunula* (34) and a *Glenodinium* sp. (11). It is also present in zooxanthellae from corals (17,21) and snails (21). 4α,24-dimethyl-5α-cholest-22E-en-3β-ol (23-desmethyldinosterol) has been identified in several dinoflagellates and zooxanthellae (10,11,19,22,23,34), in some cases as a major constituent (10,22). A similar sterol, 24-desmethyldinosterol, has been reported in 2 other dinoflagellates (5,6).

Esterified Sterols

Comparatively few reports have been made of steryl esters in dinoflagellates or other

unicellular algae although this lack may be a reflection of the analytical methods used rather than a true indication of their occurrence. In many studies, only the free sterols have been examined or the total lipids saponified so no information is obtained about the chemical forms in which the sterols occur. Our identification of esterified sterols in *G. polygramma* and their occurrence in the red-tide dinoflagellate, *Noctiluca milialis* (19), and a *Glenodinium* species (11) suggest that steryl esters may be quite common in dinoflagellates. This suggestion is certainly consistent with the finding of large amounts of steryl esters in sediments from the Black Sea where much of the organic matter originates from dinoflagellates (27). Steryl esters are common in higher plants and in some green algae (e.g., 38) but from the limited data available, they may be rare in diatoms (32,38,39). We hope that in future studies of algal sterols, more attention will be given to the chemical form in which the sterols occur, particularly as almost nothing is known about the possible occurrence of steryl sulfates or glycosides (39) in these organisms.

The major difference between the sterol composition of the free and esterified sterol fractions is the much higher proportion of 4α-methyl sterols in the latter (78 cf. 52% of total sterols). Quantitative differences also occur in the proportions of individual sterols in the free and esterified sterol fractions but these differences do not indicate major differences in the biosynthetic pathways leading to the 2 pools of sterols. Rather, in *Gonyaulax polygramma* the different structural and metabolic roles of the free and esterified sterols seem to be achieved by varying the proportion of the individual sterols and not by synthesis of new or different sterols. Considerable interspecies variation probably exists in steryl ester biosynthesis because Teshima et al. (19) found only minor differences between the compositions of free and esterified sterols in *Noctiluca milialis*, but in other algae (11,38) quite different distributions of sterols are found in the free and esterified sterol fractions.

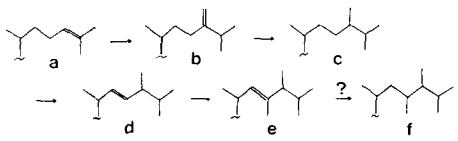
Sterol Biosynthesis by Dinoflagellates

The mechanisms by which sterols are biosynthesized by dinoflagellates have been the subject of considerable speculation, but because few labeling studies have been done, our knowledge is still limited. At least 2 mechanisms have been suggested for the formation of the unusual 23,24-dimethyl side chain found in dinosterol and sterols 7, 8, 10, 11 and 20. Withers et al. (13) presented evidence based on labeling studies for the sequence of side chain in Scheme 1.

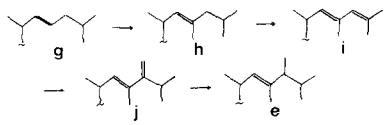
In this mechanism, the Δ22 double bond, which is seen as a necessary prerequisite for introduction of the 23-methyl group, is introduced after formation of the 24-methyl group. However, Djerassi (40) has suggested that bioalkylation of an isolated side-chain double bond can occur even when a 24-methyl substituent is not present, giving rise to a 23-methyl-Δ22-sterol. An alternative sequence of side-chain modification (Scheme 2) needs to be considered.

Of the 2 pathways, that proposed by Withers et al. (13) accounts for all of the major 4-desmethyl and 4α-methyl sterols found in *G. polygramma*. Thus, in the 4-desmethyl sterol series, the probable sequences are (a) cholesta-5,24-dien-3β-ol (desmosterol) → cholest-5-en-3β-ol (36.2%) → cholesta-5,22E-dien-3β-ol (8.9%) and (b) desmosterol → 24-methylcholesta-5,-24(28)-dien-3β-ol (trace, see below) → 24-methylcholest-5-en-3β-ol (1.5%) → 24-methylcholesta-5,22E-dien-3β-ol (7.1%) → 23,24-dimethylcholesta-5,22E-dien-3β-ol (12.2%). Note that 24-methylcholesta-5,22E-dien-3β-ol could also arise directly from C24 methylation of cholesta-5,22E-dien-3β-ol, of which a significant concentration is present.

The key intermediate in Scheme 1 is 24-methylcholesta-5,24(28)-dien-3β-ol (24-methylenecholesterol), because its presence indicates that C24 alkylation precedes alkylation at either C22 or C23. The presence of this sterol is not immediately apparent from capillary GC of the free 4-desmethylsterols so using sensitive GC-MS techniques was necessary, as described in the Results section. Significantly, none of the intermediates predicted by Scheme 2 could be detected in *G. polygramma*, even when these GC-MS techniques were used. Either they are



SCHEME 1



SCHEME 2

not accumulated by the alga at all, or more likely, this biosynthetic pathway does not occur in *G. polygramma.*

The mechanism by which nuclear-saturated 4-desmethyl sterols (5α-stanols) are formed in dinoflagellates is not known. One possibility (12,18) is that the Δ5-sterol is first oxidized to a 3-oxo-Δ5-sterol, which rearranges to a 3-oxo-Δ4-sterol, which in turn is reduced to the 5α-stanol. At which stage in the sterol biosynthetic pathway such a process might occur is not obvious. If it occurs before side-chain modification, then the reduced sterol (presumably 5α-cholest-24-en-3β-ol) could be modified by the pathway shown in Scheme 1 to produce 5α(H)-analogs of each of the Δ5 and Δ5,22 sterols in *G. polygramma.* Alternatively, the reduction step might occur after the side-chain modified sterols are formed. Whichever is the case, the abundance of the 5α-stanol depends on the structure of the side chain and presence or absence of a Δ22 double bond. Thus, the C27 stanol, 5α-cholest-22E-en-3β-ol, was below the level of detection whereas the concentration of 23,24-dimethyl-5α-cholest-22E-en-3β-ol is almost twice that of 23,24-dimethylcholesta-5,22E-dien-3β-ol (Table 1).

The biosynthetic pathway represented by Scheme 1 also accounts for the major 4α-methyl sterols in *G. polygramma.* The probable sequence is 4α-methyl-5α-cholest-24-en-3β-ol (not detected) → 4α-methyl-5α-cholest-24(28)-en-3β-ol (1.5%) → 4α,24-dimethyl-5α-cholestan-3β-ol (trace) → 4α,24-dimethyl-5α-cholest-22E-en-3β-ol (12.7%) → 4α,23,24-trimethyl-5α-cholest-22E-en-3β-ol (29.4%) → (?) 4α,23,24-trimethyl-5α-cholestan-3β-ol (50.1%). The fully saturated 4α-methyl sterol, 4α-methyl-5α-cholestanol, could be formed by reducing the presumed precursor, 4α-methyl-5α-cholest-24-en-3β-ol, whereas 4α,23,24-trimethyl-5α-cholestan-3β-ol (dinostanol) could be formed by reducing the Δ22 double bond in 4α,23,24-trimethyl-5α-cholest-22E-3β-ol (dinosterol), although this remains unproven. Alternatively dinostanol could be formed by quenching the carbonium ion intermediate in the formation of dinosterol (8).

No Δ5 unsaturated 4α-methyl sterols were detected and yet Δ5 unsaturated sterols predominate in the 4-desmethyl sterol fraction. This implies that the initial substrate for the side-chain modification sequence is Δ5 unsaturated in the case of the 4-desmethyl sterols but saturated at C5 in the case of 4-methyl sterols. This is confirmed by our identification of the 2 key intermediates, 24-methylcholesta-5,24(28)-dien-3β-ol and 4α,24-dimethyl-5α-cholest-24(28)-en-3β-ol, but why this dichotomy exists is not clear. Our detailed analysis of the sterols in this alga has pointed out several areas where knowledge of the biosynthetic pathways involved is very limted and we hope that more biosynthetic studies to address these deficiencies, using appropriate tracer techniques, will be carried out in the future.

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REFERENCES

1. Shimizu, Y. (1978) in Marine Natural Products, Chemical and Biochemical Perspectives (Scheuer, P.J., ed.) pp. 1-42, Academic Press, New York.
2. Shimizu, Y., Alam, M., and Kobayashi, A. (1976) J. Am. Chem. Soc. 98, 1059-1060.
3. Finer, J., Clardy, J., Kobayashi, A., Alam, M., and Shimizu, Y. (1978) J. Org. Chem. 43, 1990-1992.
4. Alam, M., Sansing, T.B., Busby, E.L., Martiniz, D.R., and Ray, S.M. (1979) Steroids 33, 197-203.
5. Alam, M., Schram, K.H., and Ray, S.M. (1978) Tetrahedron Lett. 3517-3518.
6. Alam, M., Martin, G.E., and Ray, S.M. (1979) J. Org. Chem. 44, 4466-4467.
7. Wengrovitz, P.S., Sanduja, R., and Alam, M. (1981) Comp. Biochem. Physiol. 69B, 535-539.
8. Alam, M., Sansing, T.B., Guerra, J.R., and Harmon, A.D. (1981) Steroids 38, 357-382.
9. Ando, T., and Barbier, M. (1975) Biochem. Syst. Ecol. 3, 245.
10. Kokke, W.C.M.C., Withers, N.W., Massey, I.J., Fenical, W., and Djerassi, C. (1979) Tetrahedron Lett. 3601-3604.
11. Kokke, W.C.M.C., Fenical, W., and Djerassi, C. (1981) Phytochem. 20, 127-134.
12. Withers, N.W., Tuttle, R.C., Holz, G.G., Beach, D.H., Goad, L.J., and Goodwin, T.W. (1978) Phytochem. 17, 1987-1989.
13. Withers, N.W., Tuttle, R.C., Goad, L.J., and Goodwin, T.W. (1979) Phytochem. 18, 71-73.
14. Withers, N.W., Goad, L.J., and Goodwin, T.W. (1979) Phytochem. 18, 889-901.
15. Withers, N.W., Kokke, W.C.M.C., Rohmer, M., Fenical, W.H., and Djerassi, C. (1979) Tetrahdron Lett. 3605-3608.
16. Swenson, W., Tagle, B., Clardy, J., Withers, N.W., Kokke, W.C.M.C., Fenical, W., and Djerassi, C. (1980) Tetrahedron Lett. 21, 4663-4666.
17. Bohlin, L., Kokke, W.C.M.C., Fenical, W., and Djerassi, C. (1981) Phytochem. 20, 2397-2401.
18. Nichols, P.D., Volkman, J.K., and Johns, R.B.

(1983) Phytochem. 22, 1447-1452.
19. Teshima, S., Kanazawa, A., and Tago, A. (1980) Mem. Fac. Fish., Kagoshima Univ. 29, 319-326.
20. Trench, R.K. (1981) Pure Appl. Chem. 53, 819-835.
21. Steudler, P.A., Schmitz, F.J., and Ciereszko, L.S. (1977) Comp. Biochem. Physiol. 56B, 385-392.
22. Kokke, W.C.M.C., Fenical, W., Bohlin, L., and Djerassi, C. (1981) Comp. Biochem. Physiol. 68B, 281-287.
23. Kokke, W.C.M.C., Bohlin, L., Fenical, W., and Djerassi, C. (1982) Phytochem. 21, 881-887.
24. Kelecom, A., Sole Cava, A.M., and Kannengiesser, G.J. (1980) Bull. Soc. Chem. Belg. 89, 1013-1014.
25. Steidinger, K.A. (1968) Florida Board of Conservation Leaflet, Series 1, 1-5.
26. Boon, J.J., Rijpstra, W.I.C., De Lange, F., Yoshioka, M., and Shimizu, Y. (1979) Nature 267, 125-127.
27. De Leeuw, J.W., Rijpstra, W.I.C., Schenck, P.A., and Volkman, J.K. (1983) Geochim. Cosmochim. Acta. 47, 455-465.
28. Guillard, R.L., and Ryther, J. (1962) Can. J. Microbiol. 8, 229-239.
29. Gagosian, R.B., and Heinzer, F. (1979) Geochim. Cosmochim. Acta 43, 471-486.
30. Wakeham, S.G., and Frew, N.M. (1982) Lipids 17, 831-843.
31. Volkman, J.K., Gillan, F.T., Johns, R.B., and Eglinton, G. (1981) Geochim. Cosmochim. Acta 45, 1817-1828.
32. Volkman, J.K., Eglinton, G., and Corner, E.D.S. (1980) Phytochem. 19, 1809-1813.
33. Volkman, J.K., Smith, D.J., Eglinton, G., Forsberg, T.E.V., and Corner, E.D.S. (1981) J. Mar. Biol. Assoc. U.K. 61, 509-527.
34. Kokke, W.C.M.C., Fenical, W., and Djerassi, C. (1982) Steroids 40, 307-318.
35. Ando, T., Kanazawa, A., Teshima, S., and Miyawaki, H. (1979) Mar. Biol. 50, 169-173.
36. Kanazawa, A., Teshima, S., Ando, T., and Tomita, S. (1974) Bull. Jap. Soc. Scient. Fish. 40, 729.
37. Ling, N.C., Hale, R.L., and Djerassi, C. (1970) J. Am. Chem. Soc. 92, 5281-5282.
38. Ballantine, J.A., Lavis, A., and Morris, R.J. (1979) Phytochem. 18, 1459-1466.
39. Kates, M., Tremblay, P., Anderson, R., and Volcani, B.E. (1978) Lipids 13, 34-41.
40. Djerassi, C. (1981) Pure Appl. Chem. 53, 873-890.
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[Received October 19, 1983]

ERRATUM

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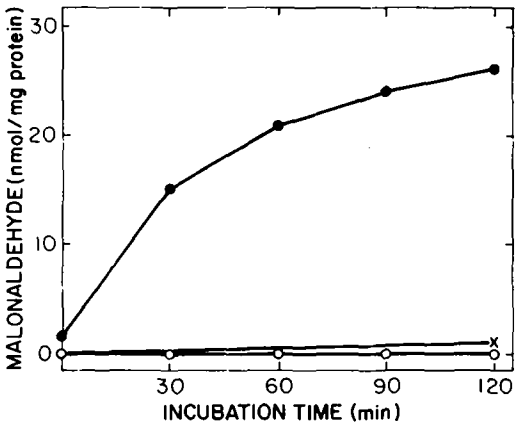

FIG. 2. Cu(II)-catalyzed peroxidation in irradiated membranes. Erythrocyte membranes were irradiated for 10 min, then incubated with (•) or without (○) 50 μM $CuSO_4$, as described under Experimental Procedures. Malonaldehyde produced during incubation with Cu(II) and either membranes irradiated in the absence of hematoporphyrin or nonirradiated membranes with hematoporphyrin is represented by (x).

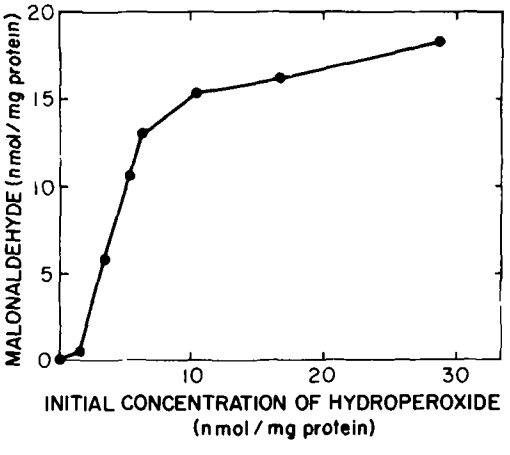

FIG. 3. Cu(II)-catalyzed malonaldehyde production as a function of initial hydroperoxide concentration. Erythrocyte membranes were irradiated at 8 W/m^2 for varying lengths of time. Hydroperoxide content was determined and duplicate irradiated samples were incubated with 50 μM $CuSO_4$ for 30 min. Malonaldehyde formed during the incubation of irradiated membranes with $CuSO_4$ was determined.

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20. Trench, R.K. (1981) Pure Appl. Chem. 53, 819-835.
21. Steudler, P.A., Schmitz, F.J., and Ciereszko, L.S. (1977) Comp. Biochem. Physiol. 56B, 385-392.
22. Kokke, W.C.M.C., Fenical, W., Bohlin, L., and Djerassi, C. (1981) Comp. Biochem. Physiol. 68B, 281-287.
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24. Kelecom, A., Sole Cava, A.M., and Kannengiesser, G.J. (1980) Bull. Soc. Chem. Belg. 89, 1013-1014.
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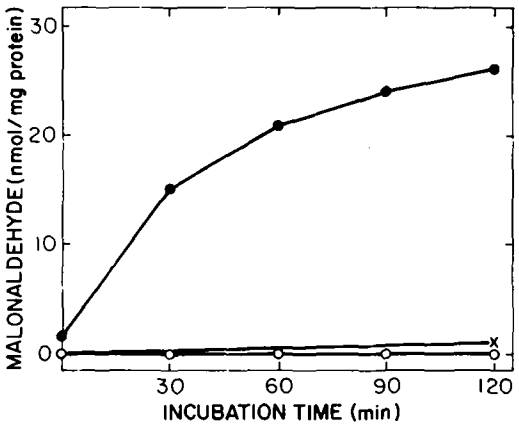

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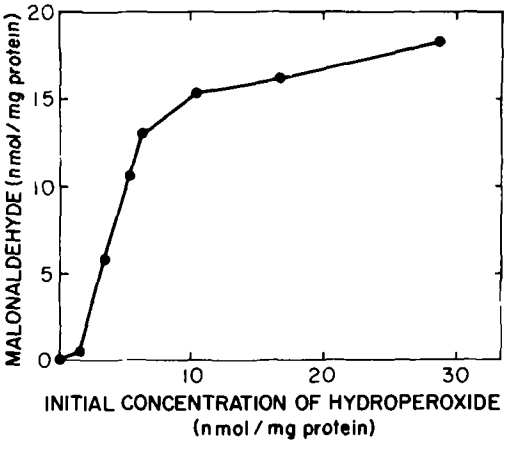

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METHODS

Hydrogenation of Unsaturated Fatty Esters During Isobutane Chemical Ionization Mass Spectrometry

WILLIAM K. ROHWEDDER* and SANDRA M. COX, *Northern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Illinois 61604*

ABSTRACT

Hydrogenation of double bonds was observed to occur during the isobutane chemical ionization mass spectrometry (MS) of unsaturated fatty esters. Chemical ionization (CI) spectra of a series of methyl esters in the C16 - C20 carbon range containing 0 - 4 double bonds showed a variety of ionization characteristics in the molecular ion cluster, including hydride abstraction, charge exchange, protonation and, for the unsaturated fatty acids (FA), hydrogenation of the double bond followed by protonation.
Lipids 19:466-468, 1984.

INTRODUCTION

Isobutane chemical ionization (CI) provides an excellent mass spectrometry (MS) technique for qualitative and quantitative evaluation of the molecular ion region of methyl esters of fatty acids (FA). Chemical ionization MS of esters with a variety of reagent gases has been studied since 1966 (1). Tsang and Harrison studied the change in the nature of CI with the change in the chain length of saturated methyl esters (2). Others have studied the possibility of determing double-bond location with CI using hydroxy derivatives (3), and techniques have been reported for the analysis of FA mixtures (4). Recently, the usefulness of CI in the analysis of substituted fatty acids has been demonstrated (5).

Data presented here show that spectra of unsaturated methyl esters produced during chemical ionization with isobutane reagent gas have a peak at mass M+3, which corresponds to hydrogenation of the double bond followed by protonation. This extra peak must be considered in both qualitative and quantitative work because it falls at the same mass as compounds having one less double bond or a label containing 2 deuterium atoms. The standard samples, the procedure and the data presented here, were part of a project to analyze lipids derived from human subjects fed deuterium-labeled fats (6,7).

EXPERIMENTAL

The test sample, a mixture of the 10 methyl esters (methyl palmitoleate [16:1], methyl palmitate [16:0], methyl linoleate [18:2], methyl oleate [18:1], methyl stearate [18:0], methyl arachidonate [20:4], methyl eicosatrienoate [20:3], methyl eicosadienoate [20:2], methyl eicosenoate [20:1] and methyl arachidate [20:0]), was prepared from high-purity methyl esters. One μl of isooctane solution, containing the mixture with ca. one μg of each of the compounds, was injected with a 10:1 split onto a 30m, wide-bore, thick film, fused silica capillary DBl gas chromatograph (GC) column. The GC was temperature-programmed from 176 C to 221 C at 2.3 degrees per min.

The GC-MS system was a Finnigan Model 4000 with a Finnigan INCOS 2000 data system. The exit end of the GC column was brought to within 20 mm of the ion beam, and isobutane was bled into the system through an auxiliary port in the separator oven. The isobutane pressure was measured by the Finnigan source thermocouple gauge, and the source temperature was held at 140 C. The 10-component test mixture was completely separated on the GC column, and the intensity values of the molecular ion regions were measured with a 3-stage INCOS multiple ion detection (MID) descriptor covering the 16:1 and 16:0 from mass 267 to mass 274; 18:2, 18:1 and 18:0 from mass 293 to mass 302; and 20:4 to 20:0 from mass 318 to mass 329. With these MID descriptors, the M−1 peak of 20:4 was not collected. Six replicates were recorded at 3 isobutane reagent gas pressures, 0.11, 0.21 and 0.40 torr, with an average relative standard deviation of 0.05.

RESULTS AND DISCUSSION

The intensities of the molecular ion cluster for 10 methyl esters, ionized with isobutane as the reagent gas, are shown in Table 1. The letter M in the table refers to the molecular weight of the compound, with M−1 referring to the molecular ion less 1 hydrogen atom and M+1, M+2 and M+3 to the molecular ion with 1, 2 or 3 added hydrogen atoms. The data have been corrected for the carbon 13

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*To whom correspondence should be addressed.

TABLE 1

Isotope-Corrected Relative Intensities of the Molecular Ion Region of Long-Chain Methyl Esters Ionized with Isobutane Reagent

Compound	Torr	M−1	M	M+1	M+2	M+3
16:1	0.11	2.3	3.6	93.1	0.4	0.4
M=268	0.21	1.8	2.6	93.4	0.3	1.7
	0.40	1.9	2.9	89.4	0.5	4.8
16:0	0.11	10.2	3.1	85.8	0.7	0.0
M=270	0.21	5.3	1.4	92.3	0.8	0.0
	0.40	3.0	1.8	93.9	1.0	0.1
18:2	0.11	8.5	15.7	73.8	0.3	1.5
M=294	0.21	4.5	6.9	83.5	0.5	4.4
	0.40	3.6	5.1	78.9	1.3	10.3
18:1	0.11	3.5	3.6	91.6	0.2	0.9
M=296	0.21	2.9	2.6	91.3	0.3	2.9
	0.40	3.5	2.6	85.2	0.7	7.7
18:0	0.11	11.0	3.3	85.6	0.0	0.0
M=298	0.21	5.6	1.4	92.8	0.0	0.0
	0.40	3.2	1.6	94.9	0.0	0.0
20:4	0.11	—	5.2	91.6	1.0	2.1
M=318	0.21	—	3.4	88.3	1.3	6.8
	0.40	—	4.0	77.3	3.3	13.8
20:3	0.11	6.6	8.7	82.3	0.5	1.8
M=320	0.21	4.1	4.5	84.0	0.7	6.5
	0.40	3.9	4.6	74.7	2.9	12.4
20:2	0.11	—	17.3	79.4	0.4	2.7
M=322	0.21	—	7.3	85.2	0.6	6.6
	0.40	—	5.7	80.2	1.5	11.7
20:1	0.11	5.1	3.5	90.2	0.0	0.9
M=324	0.21	4.3	2.5	89.5	0.0	3.5
	0.40	5.1	2.6	82.7	0.6	8.5
20:0	0.11	14.0	4.3	81.7	0.0	0.0
M=326	0.21	7.2	1.7	90.7	0.4	0.0
	0.40	4.3	1.8	93.2	0.5	0.2

isotope contribution, and thus the table represents the relative amounts of each ion type present in the spectrum. The 20:2 M−1 peaks are not shown because that mass and GC position are dominated by an interfering peak, probably caused by a positional isomer of 20:3.

The largest peak in all combinations of sample and reagent gas pressure is the M+1 peak, formed by protonation of the molecule. All compounds also show a molecular ion peak generated by charge exchange, and a peak at M−1 formed by hydride abstraction or removal of a hydrogen atom from the molecule by the reagent gas. Protonation, charge exchange and hydride abstraction are all expected from literature reports of chemical ionization.

Along with the expected ion types, a new ion type was observed with a mass peak of M+3 that can best be explained by hydrogenation of the double bond of the unsaturated acid esters followed by protonation. The intensities in the M+3 column show a considerable variation, being essentially zero for the saturated esters and as high as 13.8% for 20:4 at 0.40 torr reagent gas pressure. The intensity of the hydrogenation peak at M+3 increases with the number of double bonds, being most intense in the 20:4. The M+3 peak also varies with reagent gas pressure and becomes quite small at 0.11 torr—which is about the lowest pressure at which the mass spectrometer will operate in a CI mode—to greater than 10% at 0.40 torr, which is about the highest pressure the vacuum system will tolerate. No double hydrogenation M+5 peaks of polyunsaturated esters were found. This is as would be expected, because the largest M+3 peak is only 13.8%, and it would be the starting material for the double hydrogenation.

Most of the spectra described in the literature are

of saturated or substituted FA, and none show detailed molecular ion cluster peaks of isobutane CI spectra. Budzikiewicz and Busker have studied unsaturated alkenes and have provided much of the information on hydride abstraction, charge exchange and protonation of unsaturated compounds (8). They did not report hydrogenation, although one spectrum of octadecene in their paper has a significant M+3 peak.

The saturated methyl esters show hydride abstraction peaks (M−1 in the table) of 10, 11 and 14% at the lowest reagent gas pressure. These peaks are smaller at higher gas pressures. Full-scan spectra of the isobutane reagent gas shows a mass 43 peak ($C_3H_7^+$, isopropyl free radical) of 2% for 0.4 torr, 7% for 0.2 torr and 15% for 0.11 torr, which has the same trend as the intensities of 3.2, 5.6 and 11.0 for the hydride abstraction peaks of 18:0. The isopropyl free radical appears to remove a hydrogen atom from the saturated hydrocarbon chain to produce the M−1 ion and propane. The monoene esters, which have M−1 intensities under 5% throughout the reagent pressure range, appear not to be attacked significantly by the isopropyl free radical. The isopropyl free radical, which should be a good hydride abstraction reagent, appears to have a much greater affinity for the saturated chain rather than the unsaturated chain.

The 18:2 and 20:2 are both unusual because they have molecular ion peaks of 15.7% and 17.3%, respectively, at 0.11 torr. The 20:3 has a molecular ion peak half as intense as the 20:2, and all other compounds have only moderately small molecular ion peaks. This peaking of the intensity of the diene compounds compared with the other unsaturated compounds is an interesting point and future work might determine if double-bond position has an effect.

CI is used in both qualitative and quantitative work in order to emphasize the molecular ion region with the formation of the protonated molecule, which is much more stable and intense than the odd electron molecular ion formed in electron impact spectra. The M−1 and M+3 peaks found in methyl ester spectra can confuse interpretation of qualitative spectra, giving false positives for the number of double bonds present. Careful control of reagent gas pressure and frequent standards are necessary to use CI for quantitative work to correct for the presence of the M−1 and M+3 peaks.

REFERENCES

1. Munson, M.S.B., and Field, F.H. (1966) J. Am. Chem. Soc. 88(19), 4337-4345.
2. Tsang, C.W., and Harrison, A.G. (1975) J. Chem. Soc. Perkin Trans. II 1975, 1718.
3. Araki, E., Ariga, T., and Murata, T. (1976) Biomed. Mass Spec. 3, 261.
4. Murata, T., Takahashi, S., and Takeda, T. (1975) Anal. Chem. 47(3), 573-577.
5. Plattner, R.D., Gardner, H.W., and Kleiman, R. (1983) J. Am. Oil Chem. Soc. 60(7), 1298-1303.
6. Emken, E.A., Rohwedder, W.K., Dutton, H.J., Dougherty, D., Iacono, J.M., and Mackin, J. (1976) Lipids 11, 135-142.
7. Rohwedder, W.K., and Emken, E.A. (1983) 31st Annual Conference on Mass Spectrometry and Allied Topics, Boston.
8. Budzikiewicz, H., and Busker, E. (1966) Tetrahedron 36, 255-266.

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COMMUNICATIONS

A Lack of Correlation Between Linoleate and Arachidonate in Human Breast Milk

R.A. GIBSON* and G.M. KNEEBONE, *Department of Paediatrics, Flinders Medical Centre, Bedford Park 5042, South Australia, Australia*

ABSTRACT

The levels of arachidonic acid and linoleic acid found in the lipid fraction of human milk samples were compared. No correlation was found between the level of precursor linoleate and product arachidonate in 80 samples of colostrum (day 3-5) or 60 samples of mature breast milk. We attempted to test the hypothesis that the absence of a precursor-product relationship was caused by a constant level of arachidonate being secreted in the phospholipids (PL) of breast milk cells. Examination of the fatty acid (FA) composition of the PL and triglyceride fractions revealed arachidonate in high concentration in PL but that most of the total arachidonate existed in triglycerides.
Lipids 19:469-471, 1984.

INTRODUCTION

The lipid composition of breast milk has received a great deal of attention in recent years, and has been the subject of comprehensive reviews (1,2). Although the fact has been known for many years that the fatty acid composition of human breast milk varies with time of lactation (3) and diet (4), the focus of this attention has chiefly been either medium-chain saturated fatty acids (FA), or linoleic acid levels.

Recently we reported (5) the results of our analyses of the FA composition of colostrum and mature breast milk taken from Australian mothers on ad libitum diets. We now report the comparison of the linoleic acid (18:2ω6) level of colostrum and mature breast milk with the corresponding level of arachidonic acid (20:4ω6). A correlation between the levels of these 2 FA was expected because 20:4ω6 is synthesized from 18:2ω6. However, no correlation was found. This fact is significant for any dietary intervention study designed to increase the levels of long-chain polyunsaturated FA of the ω6 series in breast milk.

METHODS

Breast milk samples (1-3 ml) from 10 healthy female volunteers were collected at the early morning nursing following an overnight fast, by manual expression into plastic containers and immediately frozen. All women were on ad libitum diets and the level of nutrition was not influenced by economic factors. Samples were collected at any time during the nursing, as we have recently demonstrated (6) that this has no effect on the FA composition of milk. Collections were made from mothers either early (day 3-5, colostrum) or later (day 40-45, mature milk) in lactation.

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The lipid fraction was extracted from milk samples using 10 ml chloroform / methanol (2:1, v:v) for each 0.5 ml milk and sufficient water to result in phase separation (1.5 ml). One third of the extract was dried under nitrogen, transesterified in 1% H_2SO_4 in methanol and analyzed by gas liquid chromatography (GLC) as previously described (5). Separation of FA methyl esters was achieved on 5 ft columns of 5% SP2310 on chromosorb W AW (Supelco Inc., Bellefonte, PA) using nitrogen as a carrier gas at 20 ml·min^{-1}. The chromatographic oven was increased from an initial temperature of 125 C to 225 C at 4 C per min.

The remainder of the lipid extract was dried under nitrogen and separated into cholesterol ester, triglyceride and PL fractions on thin layer plates of silica gel H developed in a variation of the solvent system reported by Christie (7), petroleum spirit (40-60 C b.p.) / ethyl ether / acetic acid (180:30:2). These fractions were subsequently eluted from the plate, transesterified and the FA composition determined by GLC.

RESULTS AND DISCUSSION

Researchers have now confirmed, in a number of systems, including human tissues, that linoleic acid is the precursor of the ω6 series of polyunsaturated FA, including 18:3, 20:3, 20:4 and 22:4 (8). We recently reported the presence of these compounds in breast milk (6), and noted the fact that they appeared in higher concentrations in colostrum than in mature breast milk (5).

When we plotted 20:4ω6 levels against 18:2ω6, we were somewhat surprised to find that no correlation existed between the 2 for 80 samples of colostrum (Fig. 1) or 60 samples of mature breast milk (Fig. 2). A similar result can be seen in the data of Insull and Ahrens (9) for 6 in-patients and 5 out-patients on ad libitum diets.

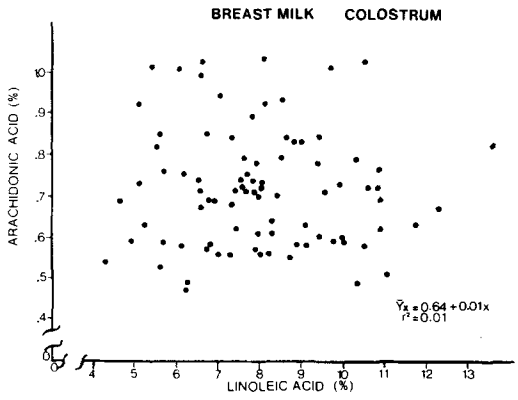

FIG. 1.

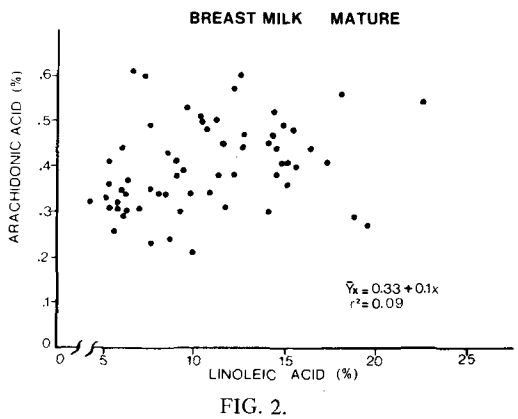

FIG. 2.

TABLE 1

Fatty Acid Distribution of Total Lipids and Triglyceride and Phospholipid Fractions of Human Colostrum

	Total	Triglycerides	Phospholipids
10:0	.33 ± .27	.34 ± .31	4.48 ± .99
12:0	2.11 ± 1.00	2.23 ± 1.18	.53 ± .33
14:0	4.75 ± .70	4.84 ± .82	2.58 ± .42
14:1	.31 ± .07	.35 ± .10	.46 ± .20
15:0	.45 ± .08	.46 ± .08	.49 ± .15
Iso 16:0	.17 ± .04	.13 ± .03	.68 ± .32
16:0	24.52 ± 1.73	23.96 ± 1.87	16.45 ± 1.73
16:1	3.68 ± .41	3.63 ± .47	1.94 ± .41
17:0	.62 ± .10	.63 ± .10	.51 ± .05
17:1	.50 ± .09	.48 ± .07	.26 ± .07
18:0	8.40 ± .75	8.16 ± .78	17.65 ± 1.49
18:1	37.31 ± 1.01	37.37 ± .90	18.49 ± 2.84
18:2ω6	8.39 ± 2.00	8.44 ± 2.21	11.03 ± 2.08
18:3ω6	.28 ± .09	.34 ± .08	.16 ± .06
18:3ω3	.37 ± .07	.41 ± .12	14 ± .09
20:0	.68 ± .11	.76 ± .16	.70 ± .23
20:1	1.13 ± .27	1.14 ± .24	.93 ± .10
20:2ω6	.76 ± .27	.63 ± .22	.44 ± .27
20:3ω6	.56 ± .25	.57 ± .19	1.51 ± .30
20:4ω6	.77 ± .26	.74 ± .14	5.76 ± 1.53
22:0	.21 ± .10	.22 ± .07	.78 ± .45
22:1	.33 ± .09	.34 ± .08	.70 ± .25
22:4ω6	.33 ± .15	.30 ± .16	1.20 ± .55
24:0	.30 ± .13	.26 ± .10	1.27 ± .77
24:1	.31 ± .11	.24 ± .11	.79 ± .69
22:5ω3	.34 ± .14	.37 ± .16	.78 ± .26
22:6ω3	.73 ± .35	.80 ± .38	1.66 ± .35

The figures represent the mean of 10 values ± SD.

A number of explanations are possible for these results. One that appeared to be readily testable was the suggestion that perhaps most arachidonic acid could be associated with PL, which, in turn, are derived from the membranes of cells secreted with the milk (10). Because the total number of cells found in breast milk alters only slowly with the length of lactation (11), the lack of correlation between the precursor linoleic acid and the product arachidonic acid could be caused by a relatively constant secretion of cellular PL.

We therefore examined the lipid fraction from the milk of 10 mothers early (day 3-5) in lactation because we had shown previously that colostrum contains the highest level of long-chain polyunsaturated FA.

The results of the GLC analyses of the FA of all fractions (i.e., total lipids, triglycerides and PL) are listed in Table 1. These data demonstrate that the level of individual FA varies in the 2 lipid fractions, and indeed the long-chair polyunsaturates, including arachidonic acid, are present in the greatest

concentration in the PL fraction. However, the PL represent only 1.7% of the combined triglyceride (97.8%), sterol ester (0.5%) and PL fractions, agreeing with the results of Crawford et al. (12). Thus, because the bulk of long-chain polyunsaturates are located in triglycerides, the higher level of arachidonic acid in colostrum, compared with mature breast milk, is not only caused by higher PL content of colostrum.

The lack of correlation between the linoleic acid and arachidonic acid levels in milk lipids is interesting, and cannot be explained without further investigation. The fact that mature breast milk can contain levels of linoleic acid ranging from 4% to 24% without concommitant changes in arachidonic acid levels indicates specific metabolic regulation of arachidonic acid. This could be achieved either by rapid use of arachidonate or control of its synthesis.

Whether these results can be related to different arachidonate pools, as has been recently postulated to occur in animals (13,14), is questionable, but they may be a reflection of use of plasma 20:4ω6 by mammary tissue. Although no evidence has been found of a metabolic block in the synthesis of 20:4ω6 in man, despite the reported slow conversion of linolenate (18:3ω3) to move unsaturated metabolites (15), some control of plasma arachidonate synthesis must occur.

A number of studies are now available that indicate that the level of arachidonate in tissues is tightly controlled in animals other than the rat. For example, we have shown that marmosets on a diet rich in 18:2ω6 accumulate linoleate in their membranes but not 20:4ω6 (16). More pertinent is the study of Mellies et al. (17) in which mothers on a diet high in linoleate did not increase arachidonate levels in either plasma or milk lipids despite large increases in 18:2ω6 in these body fluids. In contrast, Australian aboriginals on a diet rich in 20:4ω6, demonstrated elevated plasma arachidonate levels after 2 weeks (18). Taken together these results suggest that increasing 20:4ω6 in breast milk or other tissues by increasing dietary linoleate will have little effect. Whether it can be achieved by increasing dietary ω6 long-chain polyunsaturates themselves is currently being tested.

ACKNOWLEDGEMENTS

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REFERENCES

1. Jensen, R.G., Hagerty, M.M., and McMahon, K.E. (1978) Am. J. Clin. Nutr. 31, 990-1016.
2. Jensen, R.G., Clarke, R.M., and Ferris, A.M. (1980) Lipids 15, 345-355.
3. Read, N.W.C., and Sarrif, A. (1965) Am. J. Clin. Nutr. 17, 177-179.
4. Insull, W., Hirsch, J., James, T., and Ahrens, E.H. (1959) J. Clin. Invest. 38, 443-450.
5. Gibson, R.A., and Kneebone, G.M. (1981) Am. J. Clin. Nutr. 34, 252-257.
6. Gibson, R.A., and Kneebone, G.M. (1980) J. Nutr. 110, 1671-1675.
7. Christie, W.W. (1973) Lipid Analysis, pp. 152-175, Pergamon Press, New York.
8. Kanau, W.H., and Holman, R.T. (1977) Polyunsaturated Fatty Acids, American Oil Chemists Society, Champaign.
9. Insull, W., and Ahrens, E.H. (1959) Biochem. J. 72, 27-33.
10. Ho, F.C.S., Wong, R.L.C., and Lawton, J.W.M. (1979) Acta Paediatr. Scand. 68, 389-396.
11. Bhaskaram, P., and Reddy, V. (1981) Acta Paediatr. Scand. 70, 87-90.
12. Crawford, M.A., Sinclair, A.J., Msuya, P.M., and Munhambo, A. (1973) in Dietary Lipids and Postnatal Development, pp. 41-56, Raven Press, New York.
13. Hansen, H.S., and Jensen, B. (1983) Lipids 18, 682-690.
14. Hansen, H.S., Fjalland, B., and Jensen, B. (1983) Lipids 18, 691-695.
15. Dyerberg, J., Bang, H.O., and Aagaard, O. (1980) Lancet i, 199.
16. McMurchie, E.J., Gibson, R.A., Charnock, J.S., and McIntosh, G.H. (1984) Comp. Biochem. Physiol. (in press).
17. Mellies, M.J., Ishikawa, T.T., Gartside, P.S., Burton, K., MacGee, J., Allen, K., Steiner, P.M., Brady, D., and Glueck, C.J. (1979) Am. J. Clin. Nutr. 32, 299-303.
18. O'Dea, K., and Sinclair, A.J. (1982) Am. J. Clin. Nutr. 36, 868-872.

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The Very Long Chain Fatty Acids of the Green Alga, *Chlorella kessleri*

TOMÁŠ ŘEZANKA and **MILOSLAV PODOJIL***, *Institute of Microbiology, Czechoslovak Academy of Sciences, Prague 4, 142 20 Czechoslovakia*

ABSTRACT

Fatty acids of the fresh-water green alga, *Chlorella kessleri* (heterotrophically cultivated), were fractionated (as methyl esters) using high performance liquid chromatography (HPLC). A fraction of fatty acids with chain lengths longer than 28 carbon atoms was analyzed by gas chromatography-mass spectrometry (GC-MS). The very long chain fatty acids, ranging from 31 to 36 carbon atoms, were found for the first time in a green alga.
Lipids 19:472-473, 1984.

INTRODUCTION

In our previous communication (1), we described the distribution of fatty acids of a collection of fresh-water green algae, e.g., fatty acids with up to C_{30} chain lengths in *Chlorella kessleri* cultivated heterotrophically. The detector response indicated the presence of higher homologues but they could not be precisely analyzed because of the low proportion.

This study was aimed at separating a fraction of very long chain fatty acids with more than 28 carbon atoms from the whole complex of fatty acids and determining the composition of fatty acids in this fraction.

EXPERIMENTAL METHODS

A sample of fatty acids (3,500 mg) from the fresh-water green alga, *C. kessleri*, was converted to methyl esters by boron trifluoride-methanol reagent (2).

High performance liquid chromatography (HPLC) of fatty acids was performed using a SP 8000 instrument (Spectra Physics, USA) with a Separon SI C1 column 50 cm × 6 mm i.d. (Laboratorní přístroje, ČSSR); mobile phase-linear gradient (2 ml/min) from the mixture methanol/water (80:20) into methanol (30 min), followed by methanol (60 min), then detection in a UV detector at 210 nm. The column efficiency was 8,700 theoretical plates, V_0 4 ml and t_R of the methyl ester of octacosanoic acid (28:0) was 27.13 min. In 7 runs (500 mg each), 3,500 mg of a mixture of very long chain fatty acids was chromatographed. The separation was achieved by collecting fractions with retention time of more than 27.13 min and pooling fractions from twice repeated HPLC. After evaporation of the mobile phase, the total yield of a mixture of fatty acids with the chain longer than 28 carbon atoms was 32.2 mg.

*To whom correspondence should be addressed.

These were separated and identified on a Hewlett-Packard 5992 B (USA) gas chromtograph-mass spectrometer (GC-MS) instrument with a SCOT glass capillary column (SGE, Australia) 77 m × 0.5 mm i.d., with SE-30 stationary phase. The operating conditions of the instrument were: ionization energy 70 eV, scan speed 690 amu/s, mass range 4-600 amu; data were processed with a HP 9825 A computer connected online with the GC-MS. For the separation of very long chain fatty acids, the capillary column oven temperature was programmed from 100-290 C (2 min isothermally, ballistically to 240 C, then at a rate of 2C/min to 290 C, and further isothermally 50 min). The flow of carrier gas (helium) was 1.5 ml/min. The t_R of methyl ester of octacosanoic acid was 9.15 min and that of the methyl ester of hexatriacontenoic acid (36:1) 57.21 min.

RESULTS AND DISCUSSION

The usual fatty acids of the fresh-water green algae (regardless to branching) range from C_{14} to C_{22} (3). Fatty acids with longer chains have been described in mycobacteria (up to 70 carbon atoms) (4); *Saccharomyces cerevisiae* (5,6); marine algae, *Botryococcus braunii* (7) and *Emiliana huxleyi*, (unsaturated fatty acids 36:2 and 36:3) (8); higher plants (fatty acid esters up to 60 carbon atoms) (9,10).

Table 1 shows the percentage representation of very long chain fatty acids in the whole complex of fatty acids of *C. kessleri*. The fatty acids found were saturated and monounsaturated from C_{29} up to C_{36}, odd and even numbered, most of them with a straight chain. Only one fatty acid (C_{29}) was branched. The ratio of saturated and unsaturated fatty acids changes with the number of carbons; in the C_{29} acids, the saturated fatty acids predominate; in the C_{30} acids, the unsaturated acid predominates and in the higher homologues, the saturated fatty acids are missing.

TABLE 1

Percentages of Very Long-chain Fatty Acids of the Alga, *C. kessleri* (Heterotrophically Cultivated), and MS Fragments Imp tant for Identification

Methyl ester	Percentage[a]	M	M-29	M-31	M-32	Other ions			Base peak
29:0 br_2	3.6	452(13)	423(1)	–	–	381(1)	325(1)	269(2)	88
29:1	6.3	450(1)	–	419(7)	418(18)	376(2)[b]	334(2)[c]	74(43)	55
29:0	9.9	452(17)	423(1)	421(1)	–	367(1)	311(1)	255(2)	74
30:1	60.0	464(1)	435(1)	433(5)	432(5)	390(1)[b]	348(1)[c]	311(1)	55
30:0	17.0	466(20)	437(1)	435(1)	–	423(4)[d]	367(2)	311(1)	74
31:1	3.9	–	–	–	446(13)	404(2)[b]	362(2)[c]	199(2)	55
32:1	14.6	–	–	461(5)	460(10)	418(1)[b]	376(1)[c]	199(2)	55
33:1	1.8	–	–	475(3)	474(5)	432(2)[b]	199(7)	143(10)	55
34:1	2.6	–	–	489(3)	488(6)	199(8)	143(12)	74(56)	55
35:1	2.0	–	–	503(2)	502(3)	199(7)	143(8)	74(32)	55
36:1	2.1	–	–	517(1)	516(3)	199(3)	143(12)	74(58)	55

[a]In the whole complex of fatty acids (10^{-2}).
[b]Ion M-74.
[c]Ion M-116.
[d]Ion M-43; br_2 –branching on carbon C_2; figures in parenthesis–percentage of intensity of the base peak.

The unsaturation of very long chain fatty acids might improve the transport across the membrane in Saccharomyces, as described by Welch and Burlingame (5), by bringing down the melting point; for example, the fatty acid with one double bond melts 22 C lower than the corresponding saturated acid. The difficulty in analyzing the very long chain fatty acids in the whole complex of fatty acids was eliminated by separating lower fatty acids (up to C_{28}) using the HPLC method. Even though the proportion of very long chain fatty acids (higher then C_{30}) drops dramatically, under the experimental conditions given, the C_{36} is also detectable although only 10^{-2}% of total fatty acids.

Table 1 also shows that in monounsaturated methyl esters of fatty acids above C_{31}, the molecular ions were not found. For their determination, the ions M-31 (M-MeO) and M-32 (M-MeOH) become important, as well as the ions M-74 and M-116. The intensity of the latter goes down with the increasing number of carbon atoms. The common peak, m/z 55 (C_4H_7) (11), was found in all monounsaturated methyl esters of fatty acids.

We preferred the identification of fatty acids as methyl esters rather than TMS derivatives for the following reason: at the operating column temperature (280 C), perceptible evaporation of stationary phase (methyl siloxane) already takes place, making the employment of the SIM method for ion 73 impossible (it originates by decomposition of stationary phase, SE-30). The use of SIM method for m/z 74 (MacLafferty rearrangement of methyl ester of fatty acid) enabled scanning the spectrum in the moment of maximum ion current.

The data summarized in Table 1, and by Řezanka et al. (1), indicate that the content of very long chain fatty acids was influenced by the mode of cultivation. In the autotrophically cultivated *C. kessleri*, only fatty acids up to C_{28} were found. Under these conditions the elongation of the carbon chain probably does not take place.

REFERENCES

1. Řezanka, T., Slavíček, J., Vokoun, J., and Podojil, M. (1983) J. Chromatogr. 268, 71-77.
2. Metcalfe, L.D., and Schmitz, A.A. (1961) Anal. Chem. 33, 363-364.
3. Wood, B.J.B. (1974) in Algal Physiology and Biochemistry (Stewart, W.D.P., ed.) pp. 236-265, Blackwell Scientific Publications, Oxford, England.
4. Lechevalier, M.P., Horan, A.C., and Lechevalier, H. (1971) J. Bacteriol. 105, 313-318.
5. Welch, J.W., and Burlingame, A.L. (1973) J. Bacteriol. 115, 464-466.
6. Nurminen, T., and Suomalainen, H. (1971) Biochem. J. 125, 963-969.
7. Douglas, A.G., Douraghi-Zadeh, K., and Eglinton, G. (1969) Phytochemistry 8, 285-293.
8. Volkman, J.K., Smith, D.J., Englinton, G., Forsberg, T.E.V., and Corner, E.D. (1981) J. Mar. Biol. Assoc. U.K. 61, 509-527.
9. Kolattukudy, P.E. (1970) Lipids 5, 259-275.
10. Kleiman, R., Earle, F.R., and Wolf, I.A. (1969) J. Am. Oil Chem. Soc. 46, 505.
11. Odham, G., and Stenhagen, E. (1972) in Biochemical Applications of Mass Spectrometry (Waller, G., ed.) pp. 211-227, Wiley Interscience, New York.

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Cholesteryl Sulfate-Phosphatidylcholine Interactions

C. LE GRIMELLEC,* A. DAIGNEAULT, G. BLEAU and K.D. ROBERTS, *Department of Biochemistry, Membrane Transport Group of the University of Montreal, Montreal, Quebec, Canada, Maisonneuve-Rosemont Research Center, 5415 Boul. de l'Assomption, Montreal, Quebec, Canada H1T 2M4*

ABSTRACT

The effect of cholesteryl sulfate, a natural membrane component, on the physical state of dipalmitoyl phosphatidylcholine multilamellar vesicles was investigated using fluorescence polarization and differential scanning calorimetry techniques. Cholesteryl sulfate increased the order of acyl chains for those temperatures higher than the gel-to-liquid crystalline transition temperature while it decreased the order for those temperatures below the phase transition temperature. At equimolar concentrations, cholesteryl sulfate suppressed the crystal liquid-to-gel phase transition of dipalmitoyl phosphatidylcholine. These data suggest that sterol sulfates may provide new tools for the elucidation of molecular mechanisms involved in sterol-lipid interactions.

Lipids 19:474-477, 1984.

INTRODUCTION

Cholesteryl sulfate (Chol So_4) is a membrane component that is widely distributed in nature and its isolation has been reported from both invertebrate (1) and mammalian tissues (2,3). Although it generally represents a small percentage of total membrane sterols, Chol SO_4 has a protective effect against osmotic shock of the erythrocyte membrane (3) and may be involved in membrane modifications of the spermatozoa (4). Researchers have also suggested that Chol SO_4 could be involved in ion transport (5). In contrast to the relationships between cholesterol and phospholipids (PL), which have been extensively studied (6-10), no data are available concerning the interaction of Chol SO_4 with PL.

In the present study we have demonstrated, using fluorescence polarization and differential scanning calorimetry techniques, that Chol SO_4 suppresses the liquid-gel phase transition of synthetic dipalmitoyl phosphatidylcholine.

MATERIALS AND METHODS

L-α-dipalmitoyl phosphatidylcholine (DPPC) was purchased from Sigma Chemical Co. (St. Louis, MO). Cholesteryl sulfate was synthesized and purified by chromatography as previously reported (11). Briefly, cholesterol was purified via the dibromide derivative and sulfurylated in dry pyridine and chlorosulfonic acid. Following partition chromatography on celite, the ammonium salt of the sterol sulfate was twice crystallized from aqueous methanol (m.p. 198-201 C) (12), and solutions were prepared in chloroform/methanol (1:2 V/V). Cholesterol (special grade) was obtained from Applied Science Lab. (State College, PA). 1,6-Diphenyl-1,3,5-hexatriene (DPH) was purchased from Aldrich Chemicals (Milwaukee, WI). The purity of all compounds was verified by thin layer chromatography (TLC).

Appropriate aliquots of stock solutions of DPPC and cholesterol in chloroform, or Chol SO_4 in chloroform/methanol, were mixed in test tubes. The solvent was removed under a stream of nitrogen and then under high vacuum overnight. Some samples were also freeze-dried to monitor the possible occurrence of a phase separation during the sample preparation. Both preparations provided identical results. For the fluorescence polarization experiments, DPH in a 2 mM stock solution in tetrahydrofuran was directly added to the mixtures before evaporation. The concentration of DPH was maintained constant at 1/1000 relative to the PL concentration. Lipid suspensions were prepared either in distilled water or in 50 mM Na phosphate buffer, pH 7.2, as previously described (13). Measurements were performed on a SLM 4000 apparatus equipped with a 4-cell thermostated compartment and a magnetic stirrer. A Nesslab temperature programmable circulatory bath was connected to the spectrofluorometer and the temperature was monitored with a thermolinear probe placed directly within the cell compartment. Light scattering was reduced to very low levels by the use of cut-off filters. In all conditions, the individual values obtained were the mean of at least 4 successive measurements which, by themselves, were the average of 10 determinations. The results of the steady-state depolarization experiments are expressed in terms of fluorescence anisotropy r, with $r = I\| - I\perp G / I\| + 2I\perp G$ where $I\|$ and $I\perp$ are the fluorescence intensity observed with the analyzing polarizer parallel and perpendicular to the polarized excitation beam. A correction factor, G, equal to $I'\perp / I'\|$, the primes indicating excitation polarized in a perpendicular direction, was used to correct for the unequal transmission of differently

* To whom correspondence should be addressed.

ABBREVIATIONS

DPH: 1,6-diphenyl-1,3,5-hexatriene; DPPC: L-α-dipalmitoylphosphatidylcholine; Chol SO_4: cholesteryl sulfate; Chol: choleserol.

polarized light. The lipid order parameter, S_{DPH}, was calculated according to the method of Jahnig (14).

Samples prepared for differential scanning calorimetry were hydrated with 80% water or Na phosphate buffer. These solutions were preheated to 10 C above the theoretical phase transition of the pure PL, transferred to standard aluminum sample pans and scanned at least twice at a rate of 5 C or 10 C/min using a DSC-1B Perkin-Elmer instrument. The extent of hydrolysis of the sterol sulfate during the course of the experiments was verified by the addition of ^{14}C-cholesteryl sulfate as internal standard. Following TLC and the assay of radioactivity, the degree of hydrolysis was found to be less than 1%.

RESULTS AND DISCUSSION

Because DPH does not partition strongly in favor of domains of different lipid composition or physical state (10), the measurement of its steady-state fluorescence anisotropy provides a rapid and sensitive index of the average of changes in the order of membrane lipids (14).

In lipid suspensions composed of DPPC-Chol SO_4 at various molar ratios, Chol SO_4 increased the anisotropy of DPH, i.e., the order of acyl chains for those temperatures higher than the gel-to-liquid crystalline phase transition temperature, T_m (Fig. 1). The reverse is true for temperatures below T_m. At equimolar concentration, Chol SO_4 suppressed the liquid-gel phase transition of DPPC.

Although cholesteryl sulfate, like cholesterol (6,8,10) decreased the order for $T < T_m$, increased the order for $T > T_m$ and suppressed the transition for molar ratios of 1:1, examination of the anisotropy vs temperature curves for different molar ratios (Fig. 2) revealed that the interactions in the Chol SO_4-DPPC systems were not identical to

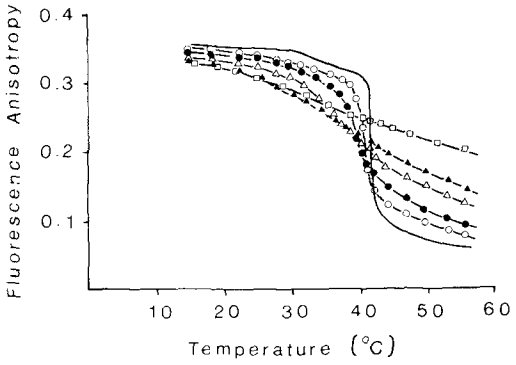

FIG. 1. Effect of increasing amounts of Chol SO_4 on the fluorescence anisotropy of DPH in DPPC suspensions. The solid line corresponds to pure DPPC. Molar concentrations of Chol SO_4 were (o) 5%, (●) 15%, (△) 25%, (▲) 33% and (□) 50%.

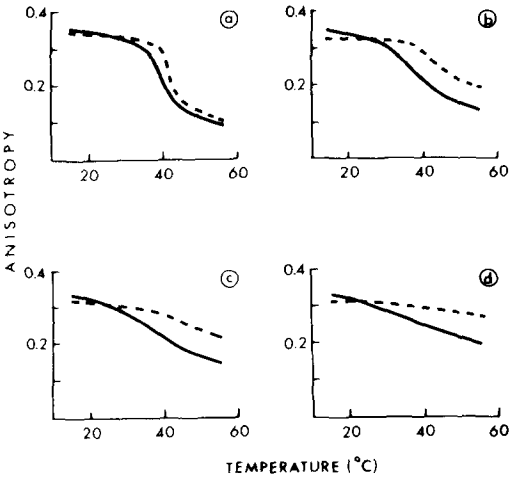

FIG. 2. Comparative effects of Chol SO_4 and cholesterol on the fluorescence anisotropy of DPH in DPPC suspensions. Molar concentrations of Chol SO_4 (full line) or cholesterol (dashed line): a: 15%, b: 25%, c: 33%, d: 50%. Note: the thickness of the lines are larger than the standard deviations of the determinations.

those found for the Chol-DPPC mixtures. Thus, the increase in order for $T > T_m$ was less pronounced than that observed with cholesterol. In particular, one does not observe the marked change that occurs between 15 - 25 mol % with cholesterol (Fig. 3a [10]). The increase in order obtained with Chol SO_4 exhibited an almost linear relationship to the fraction of the ester in the suspension. Using the same technique, other cholesterol esters, e.g., cholesteryl phosphorylcholine (15), cholesteryl hemisuccinate, cholesteryl betainate (16) and cholesteryl phosphate (17), have also been shown to increase, although to a lesser extent than cholesterol, the degree of order of egg PC liposomes or of lipids from the red blood cell. The use of DPPC enables us to demonstrate that for temperatures below the T_m, Chol SO_4 also decreased the order of acyl chains. However, for Chol SO_4/DPPC molar ratios higher than 15%, its effect was significantly more pronounced at 30 C than that of cholesterol (Figs. 2,3b) whereas the reverse was true at 20 C (Figs. 2,3c). Finally, Chol SO_4 caused a progressive shift in the transition temperature, which decreased from 41.6 C for pure DPPC to 40.4 C, 39 C, 36.3 C, and 35 C for Chol SO_4/DPPC molar ratios of 5%, 15%, 25% and 33%, respectively. Simultaneously, the transition broadened but remained detectable at 33% extending from 25 C to 45 C (Fig. 2c). The differential scanning calorimetry recording (Fig. 4) confirmed that, in contrast to cholesterol, for which very little change occurs in the midpoint temperature of the transition at increasing concentrations (6,9), Chol SO_4 produced a large downward shift in T_m. For both Chol SO_4-DPPC and

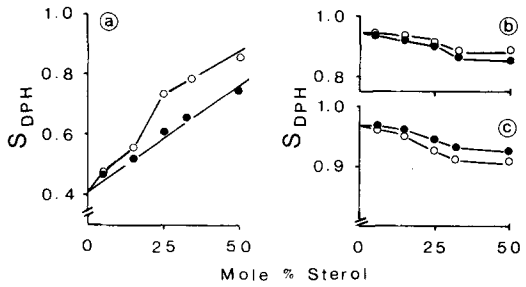

FIG. 3. Comparative effects of Chol SO_4 and cholesterol on the DPH lipid order S_{DPH}. Temperatures: 50 C (3a), 30 C (3b) and 20 C (3c); (●): Chol SO_4, (o) cholesterol.

Chol-DPPC (in control experiments) no further phase transition was detected when the relative concentration of the sterol attained 33 mol%. This value agrees with that observed by most investigators using similar equipment where detecting the presence of broad peaks is difficult (9).

The approximately linear relationship between the Chol SO_4 content of the vesicles and the decrease in transition temperature as well as the broadening of the transition suggests that part of the effects of Chol SO_4 may be caused by nonspecific interactions. In accord with classical thermodynamic theory (18), small molecules, e.g., anesthetics (19) and alcohols (20), can broaden as well as lower the transition temperature of a pure lipid. On the other hand, this factor would not account for both the fluidizing ($T < T_m$) and rigidifying ($T > T_m$) effects of Chol SO_4. The fact that equivalent amounts of cholesterol, cholesteryl sulfate and 5-α-cholestan-3-one (20,21) are required to suppress completely the gel-liquid transition would also support the importance of the sterol nucleus and of the Van der Waal's force between this nucleus and the acyl chains in this suppression. This agrees with the recent work of Bittman et al. (22) indicating that no specific or direct interaction of the PC head group with cholesterol occurs.

In conclusion, these studies demonstrated that cholesteryl sulfate, a naturally occurring cholesteryl ester, can suppress the liquid-gel transition of DPPC. It also increased the order of lipids for temperatures above the transition temperature and decreased this order for temperatures below. Although further investigation is indicated to clarify the nature of the interaction between cholesteryl sulfate and DPPC, our results suggest that sterol sulfates may represent new tools for the elucidation of molecular mechanisms involved in sterol-lipid interactions.

ACKNOWLEDGMENTS

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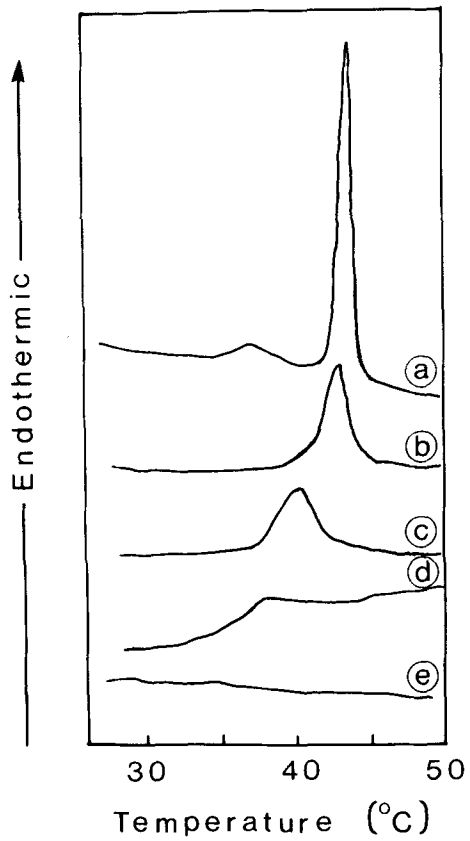

FIG. 4. DSC recordings of the effects of increasing amounts of Chol SO_4 on the transition of DPPC (heating scans). Heating rate 5 C/min. Molar concentration of Chol SO_4 (%): a: 0.0, b: 5.0, c: 13, d: 25, e: 33.

REFERENCES

1. Björkman, L.R., Karlsson, K.A., Pascher, I., and Samuelsson, B.E. (1971) Biochim. Biophys. Acta 270, 260-265.
2. Drayer, N.M., Roberts, K.D., Bandi, L., and Lieberman, S. (1964) J. Biol. Chem. 239, 3112-3114.
3. Bleau, G., Bodley, F.H., Longpré, J. Chapdelaine, A., and Roberts, K.D. (1974) Biochim. Biophys. Acta 352, 1-9.
4. Langlais, J., Zollinger, M., Plante, L., Chapdelaine, A., Bleau, G., and Roberts, K.D. (1981) Proc. Natl. Acad. Sci. USA 78, 7266-7270.
5. Abrahamsson, J., Abrahamsson, S., Hellqvist, B., Larsson, K., Pascher, I., and Sundell, S. (1977) Chem. Phys. Lipids 19, 213-222.
6. Ladbrooke, B.D., Williams, R.M., and Chapman, D. (1968) Biochim. Biophys. Acta 150, 333-340.
7. Fong, J.W., Tirri, L.J., Deshmukh, D., and Brockerhoff, H. (1977) Lipids 12, 857-862.
8. Demel, R.A., Jansen, J.W.C.M., Van Dijck, P.W.M., and Van Deenen, L.L.M. (1977) Biochim. Biophys. Acta 465, 1-10.
9. Mabrey, S., Mateo, P.L., and Sturtevant, J.M. (1978) Biochemistry 17, 2464-2468.
10. Lentz, B.R., Barrow, D.A., and Hoechli, M. (1980) Biochemistry 19, 1943-1954.
11. Roberts, K.D., Bandy, L., Calvin, H.I., Drucker, W.D., and Lieberman, S. (1964) Biochemistry 3, 1983-1988.
12. Drayer, N., and Lieberman, S. (1967) J. Clin. Endocrinol. Metab. 27, 136-139.

13. Le Grimellec, C., Giocondi, M.C., Carrière, B., Carrière, S., and Cardinal, J. (1982) Am. J. Physiol. 242, F246-F253.
14. Jahnig, F. (1979) Proc. Natl. Acad. Sci. USA 76, 6361-6365.
15. Lyte, M., and Shinitzky, M. (1979) Chem. Phys. Lipids 24, 45-55.
16. Yuli, J., Wilbrandt, W., and Shinitzky, M. (1981) Biochemistry 20, 4250-4256.
17. Colombat, A., Motta, C., Jovanel, P., Greil, J.D., Panouse-Perrin, J., Dastugve, B., and Delattre, J. (1981) Biochimie 63, 795-798.
18. Hill, M.W. (1974) Biochim. Biophys. Acta 356, 117-124.
19. Mountcastle, D.B., Biltonen, R.L., and Halsey, M. (1978) Proc. Natl. Acad. Sci. USA 75, 4906-4910.
20. Sturtevant, J.M. (1982) Proc. Natl. Acad. Sci. USA 79, 3963-3967.
21. Luken, D.W., Esfahani, M., and Devlin, T.M. (1980) FEBS Lett. 114, 48-50.
22. Bittman, R., Clejan, S., Jain, M.K., Deroo, P.W., and Rosenthal, A.F. (1981) Biochemistry 20, 2790-2795.

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Effect of Essential Fatty Acid Deficiency on Myelin Proteins

SHELDON L. MILLER*, DAVID M. KLURFELD, BERNADETTE LOFTUS and DAVID KRITCHEVSKY, *The Wistar Institute of Anatomy and Biology, 36th Street and Spruce, Philadelphia, PA 19104*

ABSTRACT

The effect of essential fatty acid (EFA) deficiency on rat-brain myelin proteins was studied. Rats were maintained on a lipid-free diet and compared with control rats fed the same diet supplemented with 3% corn oil. At 17 days of age, each pup was injected with [^{3}H]leucine and rats from each group were killed over a period of 163 days. Although a large decrease occurred in the total amount of myelin protein per brain, the proportions of constituent myelin proteins remained relatively unchanged. Metabolic studies showed a decrease in the net turnover of myelin proteins analogous to that previously demonstrated for myelin phospholipid (PL).

Lipids 19:478-480, 1984.

INTRODUCTION

Studies concerning the effects of essential fatty acid (EFA) deficiency on whole brain and myelin lipids have demonstrated: (a) changes in the acyl moieties of lipids (1-3); (b) decrease in RNA (4); (c) decrease in total protein and selected enzyme levels (4,5). Pre- and postnatal EFA deficiency was found to impair learning in rodents (6,7), and to cause motor and vision abnormalities in humans (8).

Based on the membrane model of Singer and Nicolson (9), changes in acyl composition of membrane lipids could result in changes of membrane fluidity that might alter membrane metabolism. We have previously shown that myelin phospholipid (PL) metabolism is perturbed during EFA deficiency (10). The present report is concerned with the effects of EFA deficiency on the metabolism of myelin proteins.

MATERIALS AND METHODS

Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, MA), 8 days pregnant, were fed a fat-free diet (10). Control rats received the same diet, supplemented with 3% corn oil by weight to provide EFA. Litters were reduced to 10 animals at birth, weaned at 28 days of age and the pups maintained on their respective diets for the duration of the experiment. Only male pups were used in the study.

At 17 days of age, each pup was injected intracranially with 20 μL of a solution containing 0.01 M phosphate buffer, pH 7.2, 0.9% NaCl and 750 μCi of L-[3,4,5-^{3}H]leucine (New England Nuclear, Boston, MA). One rat from the control group and one from the experimental group were decapitated at each time point (21, 45, 73, 106, 134 and 163 days) after injection. Brains were rapidly removed and myelin was isolated (11) from the forebrain, lyophilized and stored at −70 C. One aliquot was extracted for lipids (12), which were digested in perchloric acid and assayed for total lipid phosphorus (13). A second aliquot was used for lipid acyl analysis by gas liquid chromatography (GLC) (10). A third aliquot was prepared according to the method of Wiggins et al. (14) for total protein determination (15). Lipids were removed from a fourth aliquot, the protein was subjected to disc gel electrophoresis and stained with Fast Green dye (14). Gels were scanned at 570 nm on a Gilford spectrophotometer equipped with a linear transport system and the relative protein concentrations were determined from the areas under the peaks. Gels were subsequently sliced (1 mm thick), treated with Protosol (New England Nuclear), scintillation fluid was added and radioactivity was measured in a Packard Tri-Carb Model 3255 liquid scintillation spectrophotometer (14). Counting efficiency for ^{3}H was 45%.

RESULTS

EFA-deficient rats showed reduction in body weight (40%), brain weight (7%), whole brain myelin lipid phosphorus (22%) and whole brain myelin protein (25%) compared with their corresponding controls. Analysis of acyl composition showed that levels of arachidonate (20:4 [ω6]) in myelin of EFA-deficient rats were significantly lower at all time points ($P < 0.001$ by a paired Student's t test) than the corresponding controls. In the myelin of EFA-deficient rats, 20:3 (ω9) increased from 3.0% to 12.4% of the total acyl groups. The triene-to-tetraene ratio increased 4-fold in EFA-deficient rats. Acyl group 20:3 (ω9) was absent from myelin in all of the control rats except for the rat killed 163 days after injection of [^{3}H]leucine, in which it constituted only 0.1% of the total myelin acyl groups.

Figure 1 shows the percentage of several major myelin proteins in relation to the total myelin

*To whom correspondence should be addressed.

ABBREVIATIONS

EFA - essential fatty acid.

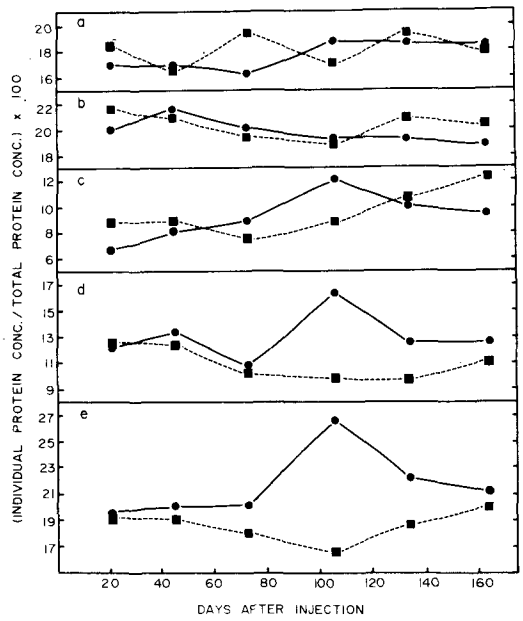

FIG. 1. Effect of EFA deficiency on myelin protein accumulation. EFA deficient (■) and control (●) rats were killed at times indicated, myelin was isolated, lipids removed and proteins separated by disc gel electrophoresis. Gels were stained with Fast Green, scanned at 570 nm and relative protein concentration determined from the area under the peak. Total protein was determined by summing the area under all protein peaks. Significance between control and EFA data was tested by trend analysis using a Student's *t* test: (a) Wolfgram protein, $P > 0.1$; (b) proteolipid protein, $P > 0.2$; (c) DM-20 protein, $P > 0.1$; (d) large myelin basic protein, $P < 0.08$; (e) small myelin basic protein, $P < 0.08$.

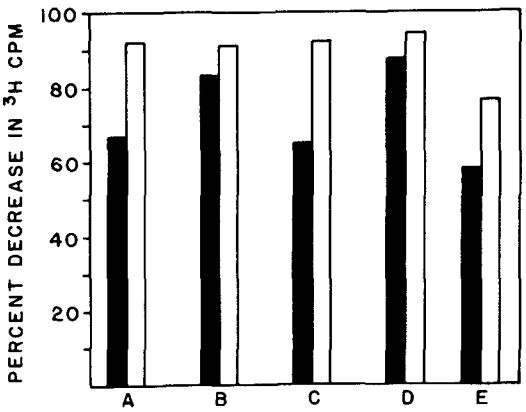

FIG. 2. Percentage decrease in ^{3}H cpm of major myelin proteins after injection with [^{3}H]leucine. EFA-deficient and control rats were injected with [^{3}H]leucine and killed at various times from 21 to 163 days after injection. Whole brain myelin was isolated from each animal, and myelin proteins were separated from each other by disc gel electrophoresis. After staining, the major myelin protein bands were cut from the gel and ^{3}H quantitated by scintillation spectrophotometry. Solid bars (EFA-deficient rats) and open bars (control rats) show the decrease in [^{3}H] protein counts in major myelin proteins from 21 to 163 days after injection. Statistical significance was determined by Student's paired *t* test as described in the Results; (a) Wolfgram proteins, $P < 0.02$; (b) proteolipid protein, $P > 0.1$; (c) DM-20 protein, $P < 0.05$; (d) large basic protein, $P < 0.1$; (e) small basic protein, $P < 0.06$. For a specific protein, differences in the ^{3}H metabolism between control and experimental data indicate the significance of the difference of the last time points.

protein content at various times after injection of EFA-deficient and control rats with [^{3}H]leucine. The experimental and control data for each protein were compared by a trend analysis using a paired Student's *t* test. The relative concentrations of Wolfgram, proteolipid and DM-20 proteins do not differ in EFA-deficient rats and controls. Large and small myelin basic proteins show a small but consistent reduction in the relative amounts of these proteins in myelin of EFA-deficient rats when compared with controls (for each protein, $P < 0.08$ by a paired Student's *t* test).

The radioactivity in individual myelin proteins from EFA-deficient and control rats was quantitated after injection of [^{3}H]leucine. Figure 2 shows the percentage of decrease of ^{3}H in the major myelin proteins from 21 to 163 days after injection. The ^{3}H loss from these myelin proteins was slower in EFA-deficient rats than in their paired controls. To determine the statistical significance between rates of ^{3}H loss from a specific protein in control and EFA-deficient animals, all values were compared by trend analysis using a paired Student's *t* test after normalization to the earliest time point. The calculated P values, based on the paired Student's *t* test, were: Wolfgram protein, $P < 0.02$; proteolipid, $P > 0.1$; DM-20 protein, $P < 0.05$; large basic protein, $P < 0.1$; small basic protein, $P < 0.06$.

DISCUSSION

The decreases in body weight, brain weight and whole brain myelin lipid phosphorus are all indicative of EFA deficiency (3,4,10). An additional indicator of EFA deficiency is a large increase in the triene/tetraene ratio of acyl moieties (16). In this study, the ratio increased 4-fold.

Myelin PL and protein, which had parallel increases, accumulated more slowly in EFA-deficient than control rats, indicating reduced deposition of myelin with a normal protein-lipid ratio. This is analogous to the alterations in myelination observed during protein-calorie malnutrition (14). The large and small basic protein of myelin are the only myelin proteins in this study whose relative amounts changed in EFA-deficient animals. Although these changes are consistent for the time

period studied, they are small in magnitude, except at one time point.

Alteration of myelin protein metabolism paralleled the PL changes seen in our previous study concerning the effects of EFA-deficiency on myelin lipids (10). The major myelin proteins—proteolipid, Wolfgram, DM-20, large and small basic proteins—showed a slower turnover rate in the EFA-deficient rats compared with their respective controls. Results from statistical analysis of the differences in 3 of these proteins were above or very slightly below the 95% confidence level. Differences between EFA-deficient and control rats in the turnover of large basic protein were not significant (probably because of some point scatter). Proteolipid showed no statistically significant change in turnover rate. Conservation of myelin protein in EFA-deficient rats is suggested by the slower loss of ^{3}H compared with controls. Because the experimental design cannot distinguish between [^{3}H]protein that remains in the membrane and [^{3}H]protein that is lost from the membrane and subsequently recycled back into it, the loss of [^{3}H]protein from myelin represents net turnover. The effect of EFA-deficiency on myelin proteins may be an alteration of turnover, recycling or both. Thus, changes in myelin protein metabolism occur in association with changes in myelin acyl composition in EFA-deficient rats.

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REFERENCES

1. Galli, C., Trzeciak, H.I., and Paoletti, R. (1971) Biochim. Biophys Acta 248, 449-454.
2. Alling, C., Bruce, A., Karlsson, I., and Svennerholm, L. (1973) in Dietary Lipids and Postnatal Development (Galli, C., Facini, G., and Pecile, A., eds.) pp. 203-215, Raven Press, New York.
3. Sun, G.Y. (1972) J. Lipid Res. 13, 56-62.
4. McKenna, M.C., and Campagnoni, A.T. (1979) J. Nutr. 109, 1195-1204.
5. Porcellati, G. (1973) in Dietary Lipids and Postnatal Development (Galli, C., Facini, G., and Pecile, A., eds.) pp. 217-222, Raven Press, New York.
6. Caldwell, D.F., and Churchill, F.A. (1966) Psychol. Reports 19, 102.
7. Galli, C., Messeri, P., Oliverio, A., and Paoletti, R. (1975) Pharm. Res. Commun. 7, 71-80.
8. Holman, R.T., Johnson, S.B., and Hatch, T.F. (1982) Am. J. Clin. Nutr. 35, 617-623.
9. Singer, S.J., and Nicolson, G.L. (1972) Science 175, 720-731.
10. Miller, S.L., Klurfeld, D.M., Weinsweig, D., and Kritchevsky, D. (1981) J. Neurosci. Res. 6, 203-210.
11. Norton, W.T., and Poduslo, S.E. (1973) J. Neurochem. 21, 749-757.
12. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497-509.
13. Eng, L.F., and Noble, B.P. (1968) Lipids 3, 157-161.
14. Wiggins, R.C., Miller, S.L., Benjamins, J.A., Krigman, M.R., and Morell, P. (1976) Brain Res. 107, 257-273.
15. Lowry, O.H., Rosenbrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
16. Pudelkewicz, C., Seufert, J., and Holman, R.T. (1968) J. Nutr. 94, 138-146.

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Species Difference of Liver Cytosolic Fatty Acid-Binding Protein in Rat, Mouse and Guinea Pig

YOICHI KAWASHIMA, YUKA TACHIBANA, SAYURI NAKAGAWA and **HIROSHI KOZUKA,** *Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-01, Japan*

ABSTRACT

Binding properties of liver cytosolic protein for oleic acid, palmitoyl-CoA and bromosulphophthalein (BSP) were compared for rat, mouse and guinea pig. Hepatic cytosol of rat, mouse and guinea pig contained proteins with a molecular weight of ca. 12,000 and had an affinity for [1-^{14}C]-oleic acid. The concentration of fatty acid-binding protein (FABP) was almost the same in livers of the animals of the 3 species and was ca. 50 μg/mg cytosolic protein. Electrophoretic studies revealed that FABP from hepatic cytosol of rat, mouse and guinea pig, purified with affinity chromatography, are distinct from one another in terms of their charge. FABP of rat liver was capable of binding any 3 ligands–oleic acid, palmitoyl-CoA and BSP–at relatively high binding capacity. FABP of mouse liver also bound oleic acid and palmitoyl-CoA to a great extent, but its binding capacity for BSP was only one-third that of rat liver. FABP of guinea pig liver bound less oleic acid and palmitoyl-CoA than rat liver, whereas it had almost the same binding capacity for BSP as rat liver.
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INTRODUCTION

Cytosolic proteins with a relatively low molecular weigh participate in cellular translocation and metabolism of organic anions that are not very soluble in water. Fatty acid-binding protein (FABP) is localized in cytosol of various organs including liver, myocardium, intestinal mucosa, adipose tissue, skeletal muscle and kidney (1,2). This protein of rat liver has been known not only to be specific for fatty acid, but also to have high affinities for long-chain acyl-CoA and several other organic anions (3,4). Considerable evidence indicates a physiological significance of FABP in translocation and metabolism of long-chain fatty acid and their CoA esters in rat liver and small intestine (5-16), and the properties of FABP in these tissues have been well studied (17-19). However, most information about the physiological role and properties of FABP was obtained from the studies on rats. To have a general understanding about physiological significance of FABP, more information should be obtained about FABP from species other than rat. In this context, we studied binding characteristics and properties of FABP in hepatic cytosol of rat, mouse and guinea pig.

MATERIALS AND METHODS

Materials

[1-^{14}C] Oleic acid (57.0 Ci/mol) and [1-^{14}C]-palmitoyl-CoA (56.1 Ci/mol) were purchased from New England Nuclear Corp. (Boston, MA). Palmitoyl-CoA, bromosulphophthalein (BSP), bovine serum albumin and Coomasie brilliant blue were purchased from Sigma Chemical Co. (St. Louis, MO); Sephadex G-50, Sephadex G-75 and aminohexylamino-Sepharose 4B from Pharmacia Fine Chemicals (Uppsala, Sweden). All other chemicals were of analytical grade. Oleoyl-aminohexylamino-Sepharose was prepared from oleic acid and amino-hexylamino-Sepharose 4B (14).

Animals

Male Wistar rats (160-180 g), male ddY mice (30-40 g) and male Hartley guinea pigs (250-390 g) were used.

Preparation of Cytosol

Animals were decapitated. Livers were isolated and perfused with cold 0.9% NaCl until the livers turned pale with no further color change, then homogenized with 1.5 vol cold 0.25 M sucrose. Cytosol was obtained from the homogenates by differential centrifugation (14,20) Hepatic cytosol of mouse was prepared from 4 or 5 pooled livers.

Binding Assay for Oleic Acid

The amount of [1-^{14}C]oleic acid bound to FABP in cytosol was measured by the method of Ockner et al. (10) with some modification (20). The incubation mixture contained 120 nmol [1-^{14}C]oleic acid (45 nCi), 7.5 mg cytosolic protein and 3 mg Triton WR-1339 in 1.5 ml 0.154 M KCl/0.01 M potassium phosphate buffer (pH 7.4). The mixture was incubated at 37 C for 30 min. After being cooled on ice,

1 ml of the mixture was subjected to gel filtration on a Sephadex G-50 column (2.2 cm × 30 cm) equilibrated with 0.154 M KCl/0.01 M potassium phosphate buffer (pH 7.4) at 4 C and eluted with the same buffer. Fractions of 2 ml were collected. In some experiments, for further resolution of cytosolic proteins, a column of Sephadex G-75 (2.2 cm × 80 cm) was used. Fourty-eight nmol [1-^{14}C]oleic acid (0.1 μCi) in 60 μl of methylethylketone was added to an incubation mixture containing 24 mg cytosolic protein in 2.34 ml of 0.154 M KCl/0.01 M potassium phosphate buffer (pH 7.4). The mixture was incubated at 0 C for 30 min and 2.0 ml of the mixture was applied to the Sephadex G-75 column. The column had been equilibrated with 0.154 M KCl/0.01 M potassium phosphate buffer (pH 7.4) and elution was carried out with the same buffer at a flow rate of 12 ml/hr at 4 C. Fractions of 4 ml were collected. The radioactivity in the eluate was measured by a liquid scintillation spectrometer after mixing with toluene and a Triton-based scintillator.

Binding Assay for Palmitoyl-CoA

Forty nmol [1-^{14}C]palmitoyl-CoA (0.1 μCi) in 0.2 ml was added to an incubation mixture containing 20 mg cytosolic protein in 1.8 ml of 0.154 M KCl/0.01 M potassium phosphate buffer (pH 7.4) at 0 C, then 1.5 ml of the mixture was applied to a Sephadex G-75 column (2.2 cm × 80 cm). The column was equilibrated with 0.154 M KCl/0.01 M potassium phosphate buffer (pH 7.4) and elution was carried out with the same buffer at a flow rate of 12 ml/hr. Fractions of 4 ml were collected and an aliquot of the fraction was used to measure radioactivity as described above.

Binding Assay for BSP

The incubation mixture contained 3 μmol BSP and 25 mg cytosolic protein in 2.0 ml 0.154 M KCl/0.01 M potassium phosphate buffer (pH 7.4). The mixture was incubated at 0 C for 30 min and then subjected to a Sephadex G-75 column (2.2 cm × 80 cm) equilibrated with 0.154 M KCl/0.01 M potassium phosphate buffer (pH 7.4). Elution was carried out with the same buffer at a flow rate of 12 ml/hr and fractions of 4 ml were collected. BSP in the fraction was determined by measuring the absorbance at 580 nm after alkalinization with 1 N NaOH.

Measurement of FABP by an Affinity Chromatography

The concentration of FABP was determined using affinity chromatography with oleoyl-aminohexylamino-Sepharose (14).

Fatty Acid Analysis

Lipids were extracted by the method of Bligh and Dyer (21) and were separated by thin layer chromatography (TLC) of Silica gel G with petroleum ether/diethyl ether/acetic acid (80:30:1, v/v/v) as developing solvent. The area of silica gel corresponding to fatty acid was identified by means of standards. Fatty acid was isolated from the silica gel by the method of Stern and Pullman (22). Methyl esters of fatty acids were prepared and analyzed by gas liquid chromatography (23). Pentadecanoic acid was used as an internal standard for quantitation.

Other Procedures

Protein concentration was determined by the method of Lowry et al. (24) with bovine serum albumin as standard. Electrophoresis with polyacrylamide disc gel was performed according to the method of Davis (25,14). Statistical analyses were performed using Student's *t* test for 2 means.

RESULTS

Figure 1 shows elution profiles of [1-^{14}C]-oleic acid bound to cytosolic protein when cytosolic protein was mixed with [1-^{14}C]oleic acid and subjected to gel filtration with Sephadex G-75. A single peak with radioactivity appeared at the position with a molecular weight of ca. 12,000 from any hepatic cytosol of rat, mouse and guinea pig, indicating that hepatic cytosol of mouse and guinea pig also contains FABP with a molecular weight similar to that of rat liver. To determine the concentration of FABP in hepatic cytosol of the animals of the 3 species, FABP fractions obtained with gel filtration were subjected to affinity chromatography of oleoyl-aminohexyl-amino-Sepharose and the amounts of FABP were measured after removing unabsorbed protein to the affinity column. As described in Table 1, the concentrations of FABP in hepatic cytosol of rat, mouse and guinea pig were 48.2 μg/mg, 49.1 μg/mg and 45.8 μg/mg cytosolic protein. The contents of hepatic FABP of mouse and guinea pig were 124% and 73% that of rat per g liver.

The FABP, which was purified by gel filtration with Sephadex and subsequent affinity chromatography with oleoyl-aminihexylamino-Sepharose from hepatic cytosol of rat, mouse and guinea pig, was analyzed with polyacrylamide disc gel electrophoresis in a nondenatur-

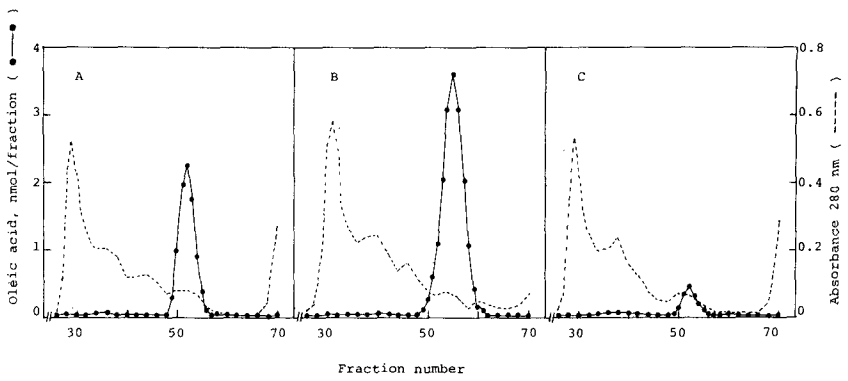

FIG. 1. [1-^{14}C] Oleic acid binding to liver cytosolic protein of rat, mouse and guinea pig. Mixture (2.4 ml) containing 24 mg of cytosolic protein was incubated for 30 min at 0 C with 40 nmol [1-^{14}C]oleic acid ad 60 μl of methylethylketone solution. Sephadex G-75 column (2.2 cm × 80 cm) was used for 2.0 ml of the mixture. A, rat; B, mouse; C, guinea pig.

TABLE 1

Concentration and [1-^{14}C] Oleic Acid Binding Capactity of Liver Cytosolic FABP of Rat, Mouse and Guinea Pig

Animals	Concentration of FABP		Oleic acid bound to FABP	
	Cytosolic protein (μg/mg)	Liver (mg/g)	Cytosolic protein (nmol/mg)	Liver (nmol/g)
Rat (5)	48.2 ± 3.5	1.74 ± 0.26	1.14 ± 0.13	40.1 ± 7.0
Mouse (3)	49.1 ± 12.2[c]	2.14 ± 0.70[c]	2.10 ± 0.08[a]	97.6 ± 6.5[c]
Guinea pig (3)	45.8 ± 7.4[c]	1.25 ± 0.31[c]	0.30 ± 0.03[a]	8.2 ± 0.5[a]

Values are mean ± SD. Values in parentheses represent the number of separate experiments. [1-^{14}C] Oleic acid binding capacity was measured by gel filtration on a Sephadex G-50 column as described in the text. Concentration of FABP was determined by affinity chromatography. FABP fractions obtained by gel filtration of cytosol were combined. An aliquot (300-900 μg of protein) of the pooled fraction was diluted with an equivolume of phosphate-bufferred saline and was applied to a column (0.9 cm × 2.5 cm) of oleoyl-aminohexylamino-Sepharose 4B. The unbound proteins to the column were washed out with 10 ml of phosphate buffered saline and the absorbed proteins were eluted with 12 ml 25% ethanol in 0.075 M sodium phosphate buffer (pH 6.0). The proteins remaining in the column were washed out with ethanol/0.05 N NaOH (1:1, v/v). The proteins eluted with 25% ethanol/0.075 M sodium phosphate buffer (pH 6.0) were regarded as FABP.

[a]$P < 0.001$ relative to rat.

[b]$P < 0.01$ relative to rat.

[c]Not significant.

ing buffer. As shown in Figure 2, the staining of the purified FABP of rat liver demonstrates the presence of more than 2 protein bands. FABP purified from mouse liver also showed more than 2 protein bands. The migration profiles in polyacrylamide gel were markedly different from those of FABP of rat liver. FABP of guinea pig liver migrated, as was observed with FABP of mouse liver, although the staining of FABP of guinea pig showed more broad bands.

The results in Figure 1 indicate that marked differences exist in the binding capacity of FABP to [1-^{14}C]oleic acid among rat, mouse and guinea pig. As described in Table 1 in more detail, the amounts of [1-^{14}C]oleic acid bound to FABP in mouse liver was ca. 2 times greater than those of rat liver. Moreover, the amounts of [1-^{14}C]oleic acid bound to FABP of guinea pig liver were one-fourth per mg cytosolic protein and one-fifth per g liver, compared with rat liver.

FABP in rat liver is known to bind various other ligands beside fatty acid (4). To compare binding capacities of FABP for ligands other than fatty acid, hepatic cytosol was mixed with BSP and was subjected to a Sephadex G-75 column. Gel filtration gave 2 major peaks of BSP associated with proteins. The peak with a molecular weight of ca. 45,000 may correspond

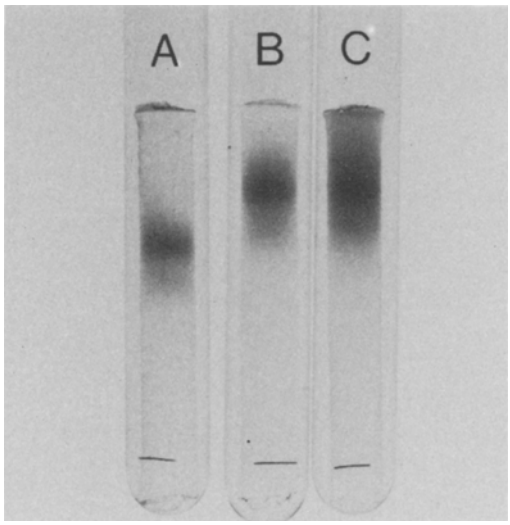

FIG. 2. Disc gel electrophoresis of FABP from liver of rat, mouse and guinea pig. FABP fractions obtained by gel filtration of hepatic cytosol were combined and subsequently applied to an oleoyl-aminohexylamino-Sepharose column (0.9 cm × 2.5 cm). The unbound proteins in the column were washed out with 10 ml of phosphate-buffered saline. The proteins bound to the gel were eluted with 12 ml of 25% ethanol in 0.075 M sodium phosphate buffer (pH 6.0). The latter proteins were regarded as purified FABP. To each gel was added ca. 20 μg of purified FABP. Polyacrylamide disc gel electrophoresis was performed as follows: 2.5% contracting (pH 6.7) and 7% separating (pH 8.9) gels were buffered with 0.5 M Tris and 0.38 M glycine (pH 8.6) and run at 0.8 mA/gel for 5 hr. Proteins were fixed and stained in 0.2% Coomasie brilliant blue in 50% methanol/7% acetic acid. A, Rat; B, mouse; C, guinea pig.

to Y-protein (ligandin) and the other peak is Z-protein with a molecular weight of ca. 12,000 (4). The amounts of BSP eluted in the 2 peaks were summarized in Table 2. Regarding Y-protein, the amounts of BSP bound to Y-protein of guinea pig were as much as 2 times greater than those of rat. Little difference was found between the amounts of BSP bound to y-protein in rat and mouse. On the other hand, no difference was found between the amounts of BSP bound to Z-protein in rat and guinea pig, whereas the amount of BSP bound to mouse Z-protein was ca. one-third that of rat Z-protein.

Because long-chain acyl-CoA has been known to be as good a ligand for FABP in rat liver as fatty acid and BSP (3), the binding pattern of [1-^{14}C]palmitoyl-CoA to cytosolic protein was analyzed with gel filtration on a Sephadex G-75 column. As shown in Figure 3, only one peak labeled with [1-^{14}C]palmitoyl-CoA appeared at the position corresponding to FABP from rat and mouse cytosol. On the other hand, 2 peaks labeled with [1-^{14}C]palmitoyl-CoA appeared from guinea pig cytosol. The 2 forms of protein capable of binding palmitoyl-CoA were eluted at the positions corresponding to molecular weights of 70,000 and 12,000. The amounts of [1-^{14}C]palmitoyl-CoA bound to the protein with higher molecular weight were 2 times greater than those bound to the protein with lower molecular weight (Table 3). When the amounts of [1-^{14}C]palmitoyl-CoA bound to the protein with a molecular weight of 12,000 are compared, binding capacity of the protein of guinea pig was one-fifth that of rat per mg cytosolic protein and one-seventh that of rat per g liver.

Liver cytosolic protein was separated by gel filtration to FABP fraction and the other fraction, and concentrations of free fatty acid in these 2 fractions were determined. As described in Table 4, FABP fraction from livers of mouse and rat contained 142.1 nmoles/g and 72.2 nmoles/g liver. These account for more than 60% of free fatty acid in hepatic cytosol of both mouse and rat. FABP fraction of guinea

TABLE 2

Binding BSP to Y-Protein and Z-Protein in Liver of Rat, Mouse and Guinea Pig

Animlas	Binding to Y-protein		Binding to Z-protein	
	Cytosolic protein (nmol/mg)	Liver (nmol/g)	Cytosolic protein (nmol/mg)	Liver (nmol/g)
Rat (3)	0.72 ± 0.09	25.8 ± 1.8	1.17 ± 0.14	41.6 ± 2.8
Mouse (3)	0.89 ± 0.21[d]	31.5 ± 8.0[d]	0.34 ± 0.01[a]	12.0 ± 1.0[a]
Guinea pig (3)	1.48 ± 0.09[b]	42.0 ± 5.2[c]	1.49 ± 0.07[c]	42.8 ± 4.1[d]

Values are mean ± SD. The number in paranthèses represent the numbers of separate experiments.
[a]P<0.001 relative to rat.
[b]P<0.01 relative to rat.
[c]P<0.05 relative to rat.
[d]Not significnat.

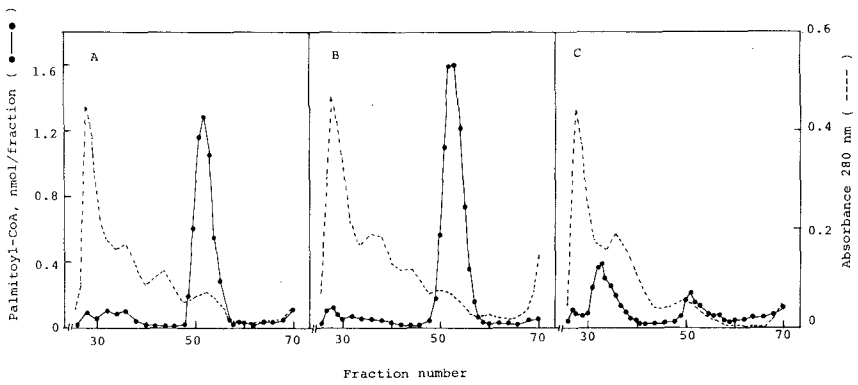

FIG. 3. Gel-filtration profiles of cytoslic protein with [1-^{14}C]palmitoyl-CoA. Fourty nmol [1-^{14}C]palmitoyl-CoA was mixed with 20 mg of cytosolic protein in 2.0 ml of 0.154 M KCl/0.1 M potassium phosphate buffer (pH 7.4) at 0 C. A Sephadex G-75 column (2.2 cm × 80 cm) was used on 1.5 ml of the mixture. A, rat; B, mouse; C, guinea pig.

TABLE 3

[1-^{14}C]Palmitoyl-CoA Binding to Liver Cytosolic Protein of Rat, Mouse and Guinea Pig

	Binding to protein with high molecular weight		Binding to protein with low molecular weight	
Animals	Cytosolic protein (nmol/mg)	Liver (nmol/g)	Cytosolic protein (nmol/mg)	Liver (nmol/g)
Rat (3)	trace	trace	0.29 ± 0.06	10.2 ± 2.1
Mouse (4)	trace	trace	0.40 ± 0.13[c]	12.1 ± 3.5[c]
Guinea pig (4)	0.13 ± 0.02	3.26 ± 0.52	0.06 ± 0.01[b]	1.4 ± 0.4[a]

Values are mean ± SD. The number in parentheses represent the number of separate experiments. High- and low-molecular weight proteins correspond to fraction numbers 30-38 and 49-58, in Figure 3.

[a]$P<0.001$ relative to rat.
[b]$P<0.01$ relative to rat.
[c]Not significant.

pig liver contained 47.2 nmoles of free fatty acid/g liver accounting for 54% of free fatty acid of hepatic cytosol of guinea pig.

DISCUSSION

Recently, the information regarding FABP of species other than rat has received attention. Haq et al. (26) reported that human adipose tissue contains FABP and that the protein has properties similar to those of FABP from rat liver. The liver of *Phatyrhindes triserata* contains Z-protein with a slight binding capacity for BSP and no binding affinity for oleic acid (27). In the present study, we provided evidence for the existence of FABP with a molecular weight of ca. 12,000 in hepatic cytosol of both mouse and guinea pig, as was observed in that of rat. Four to 5% of cytosolic protein of rat liver has been reported to be FABP (14,19). The present study showed that hepatic cytosol of mouse and guinea pig contain FABP at almost the same concentration as that of rat. These results may suggest that FABP is abundant in hepatic cytosol of any mammal. However, the properties of FABP of rat, mouse and guinea pig differed markedly in their binding capacities to ligands and in their electrical properties.

FABP from rat liver has been found to be involved with several proteins that share immunological identity but differ in electrical charge (19). Our present results also indicated that FABP purified from rat liver showed more than 2 protein bands on polyacrylamide disc gel. FABP purified from mouse liver also showed more than 2 protein bands on polyacrylamide gel, but the migration of the proteins differed from the migration of protein from rat liver. Migration of FABP from guinea pig liver was similar to that from mouse liver rather than that of rat liver, although the proteins in polyacrylamide gel were stained more broadly. The FABP were purified by affinity chromatog-

TABLE 4

Content of Endogenous Free Fatty Acid in FABP of Liver from Rat, Mouse and Guinea Pig

	FABP fraction (nmol/g liver)	Fraction other than FABP (nmol/g liver)
Rat (3)	72.2 ± 7.6	43.6 ± 4.9
Mouse (3)	142.1 ± 15.2[b]	79.4 ± 0.6[a]
Guinea pig (3)	47.2 ± 7.6[c]	41.6 ± 6.5[d]

Values are mean ± SD. The numbers in parentheses represent the numbers of separate experiments. Three ml of hepatic cytosol was subjected to a Sephadex G-75 column (2.5 cm × 45 cm) and was eluted with 0.154 M KCl/0.01 M potassium phosphate buffer (pH 7.4) at 4 C at a flow rate of 8 ml/hr. Fractions of 4 ml were collected. Fractions corresponding to FABP and fractions other than the FABP fraction were pooled separately. These 2 pooled samples were concentrated by lyophylization and lipids in the concentrated samples were extracted to analyze free fatty acid as described in the text.

[a]$P<0.001$ relative to rat.
[b]$P<0.01$ relative to rat.
[c]$P<0.05$ relative to rat.
[d]Not significant.

raphy with oleoyl-aminohexylamino-Sepharose. Therefore, these results suggest that FABP from liver of mouse and guinea pig also contain several molecular species with a similar affinity for fatty acid differing in electrical charge, as was found with FABP of rat liver.

Our present experiment has shown that FABP of mouse liver has high binding capacities for both oleic acid and palmitoyl-CoA, but the FABP has a lower binding capacity for BSP compared with rat liver. On the other hand, FABP of guinea pig liver has an extremely low binding capacity for both fatty acid and acyl-CoA, whereas it has almost the same binding capacity for BSP as that of rat. Thus, the changes in binding affinities of FABP for fatty acid and palmitoyl-CoA were not parallel to changes in affinity for BSP. Considering the fact that, on the basis of amino acid analysis of the proteins, FABP (1), Z-protein (4) and amino azo dye-bining protein (28) may be identical proteins, our present resutls may indicate that BSP and fatty acid bind at different sites of FABP.

We showed that palmitoyl-CoA was bound to 2 protein zones in hepatic cytosol of guinea pig. An immunological study with rabbit serum anti-guinea pig albumin suggests that the eluate corresponding to the peak with a high molecular weight (ca. 70,000) was contaminated by serum albumin at the level of 2-4%. On the other hand, the protein did not bind [1-^{14}C]-oleic acid, as shown in Figure 1, despite the possibility that the protein may bind [1-^{14}C]-oleic acid easily if the protein is serum albumin. Hepatic cytosol of rat and mouse did not contain the protein. Therefore, the entity of the cytosolic protein, which was demonstrated in guinea pig liver to have a molecular weight of ca. 70,000 and to be capable of binding palmitoyl-CoA, has not been clarified at the present stage.

Ockner et al. (19) reported that FABP of rat liver binds 60% of free fatty acid in cytosol. We found that FABP of rat liver binds 64% of free fatty acid in cytosol, which agrees with Ockner et al. (19). We found this was also the case for FABP of mouse liver. FABP of guinea pig liver was found to bind as much as 54% of free fatty acid in cytosol, despite its low binding capacity for oleic acid. This may be caused by the absence of a carrier of free fatty acid instead of FABP in the cytosol.

Although FABP binds various ligands, several lines of evidence suggest the relationship in vivo between the protein and either cellular translocation or use of long-chain fatty acid (5-12,14). Moreover, FABP has been demonstrated to stimulate, in vitro, several enzymic reactions involved in fatty acid metablism (7,11-13,15,16) and the high affinity of FABP for long-chain acyl-CoA is important when the protein acts as a modulator on the activity of acetyl-CoA carboxylase (29), mitochondrial adenine nucleotide translocase (30), methylsterol oxidase (31) and hydroxymethylglutaryl-CoA reductase (31). We showed the presence of FABP in hepatic cytosol of rat, mouse and guinea pig and the differences in affinites of FABP for diverse ligands among the animals of the 3 species. These results indicate that FABP may conceivably play a physiologically important role, such as mentioned above, in hepatocytes of mammals of any species.

REFERENCES

1. Ockner, R.K., Manning, J.A., Poppenhausen, R.B., and Ho, W.K.L. (1972) Science 177, 56-58.
2. Mishkin, S., Stein, L., Gatmaintan, Z., and Arias, I.M. (1972) Biochem. biophys. Res. Commun. 47, 997-1003.
3. Mishkin, S., and Turcotte, R. (1974) Biochem. Biophys. Res. Commun. 57, 918-926.
4. Levi, A.J., Gatmaitan, Z., and Arias, I.M. (1969) J. Clin. Invest. 48, 2156-2167.
5. Goresky, C.A., Daly, D.S., Mishkin, S., and Arias, I.M. (1978) Am. J. Physiol. 234, E542 E553.
6. Kushlan, M.C., Gollan, J.L., Ma, W.-L., and Ockner, R.K. (1981) J. Lipid Res. 22, 431-436.
7. Wu-Rideout, M.Y.C., Elson, C., and Shargo, E. (1976) Biochem. Biophys. Res. Commun. 71, 809-816.

8. Mishkin, S., Stein, L., Fleischner, G., Gatmaitan, A., and Arias, I.M. (1975) Am. J. Physiol. 228, 1634-1640.
9. Ockner, R.K., Burnett, D.A., Lysenko, N., and Manning, J.A. (1979) J. Clin. Invest. 64, 172-181.
10. Ockner, R.K., Lysenko, N., Manning, J.A., Monroe, S.E., and Burnett, D.A. (1980) J. Clin. Invest. 65, 1013-1023.
11. Burnett, D.A., Lysenko, N., Manning, J.A., and Ockner, R.K. (1979) Gastroenterol. 77, 241-249.
12. Mishikin, S., and Turcotte, R. (1974) Biochem. Biophys. Res. Commun. 60, 376-381.
13. Applekvist, E.L., and Dallner, G. (1980) Biochim. Biophys. Acta 617, 156-160.
14. Kawashima, Y., Nakagawa, S., Tachibana, Y., and Kozuka, H. (1983) Biochim. Biophys. Acta 754, 21-27.
15. O'Doherty, P.J.A. (1975) FEBS Lett. 60, 256-258.
16. Ockner, R.K., and Manning, J.A. (1976) J. Clin. Invest. 58, 632-641.
17. Ockner, R.K., and Manning, J.A. (1974) J. Clin. Invest. 54, 326-338.
18. Takahashi, K., Odani, S., and Ono, T. (1982) FEBS Lett. 140, 63-66.
19. Ockner, R.K., Manning, J.A., and Kane, J.P. (1982) J. Biol. Chem. 257, 7872-7878.
20. Kawashima, Y., Nakagawa, S., and Kozuka, H. (1982) J. Pharmacol. Dyn. 5, 771-779.
21. Bligh, E.G., and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911-917.
22. Stern, W., and Pullman, M.E. (1978) J. Biol. Chem. 253, 8047-8055.
23. Kawashima, Y., and Kozuka, H. (1982) Biochim. Biophys. Acta 713, 622-628.
24. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
25. Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 404-426.
26. Haq, R.-U., Christodoulides, L., Ketterer, B., and Shargo, E. (1982) Biochim. Biophys. Acta 713, 193-198.
27. Sugiyama, Y., Yamada, T., and Kaplowitz, N. (1982) Biochem. J. 203, 377-381.
28. Ketterer, B., Ross-Mansell, P., and Whitehead, J.K. (1976) Biochem. J. 103, 316-324.
29. Lunzer, M.A., Manning, J.A., and Ockner, R.K. (1977) J. Biol. Chem. 252, 5483-5487.
30. Barbour, R.L., and Chan. S.H.P. (1979) Biochem. Biophys. Res. Commun. 89, 1168-1177.
31. Grinstead, G.F., Trzaskos, J.M., Billheimer, J.T., and Gaylor, J.L. (1983) Biochim. Biophys. Acta 751, 41-51.

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Analysis of the Stearoyl-CoA Desaturase System in the Morris Hepatoma 7288C and 7288CTC

RAPHAEL A. ZOELLER* and **RANDALL WOOD**, *Department of Biochemistry and Biophysics, Texas Agricultural Experiment Station, Texas A&M University System, College Station, TX 77843*

ABSTRACT

The microsomal stearoyl-CoA desaturase system was examined in both the Morris hepatoma 7288CTC, maintained in the host Buffalo strain rat, and the Morris hepatoma 7288C, maintained in tissue culture. In vitro examination shows the stearoyl-CoA desaturase system to be similar in the 2 tissues. Both show extremely low overall stearoyl-CoA desaturase activity, having 4% and 8% of normal liver values respectively. Examination of the electron transport system showed both tissues have decreased electron transport components cytochrome b_5 and cytochrome b_5 reductase. Particularly noticeable were the extremely low levels of cytochrome b_5 (2% compared with normal liver). Microsomes from both tissues showed a decreased ability to reduce an artificial electron acceptor, cytochrome c. With the low levels of cytochrome b_5 observed in these tissues, the low levels of overall desaturase activity may be caused by lack of terminal enzyme, lack of sufficient cytochrome b_5, or both. Analysis of the stearoyl-CoA desaturase system in cultured hepatoma cells suggests that these cells are similar to the host-grown tumor in this respect and may be used as a model in further examinations of the stearoyl-CoA desaturase system.
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INTRODUCTION

Differences in the lipid composition of hepatoma compared with normal liver tissue have been well documented. One commonly observed phenomenon is the increased levels of monoenoic fatty acids (1-5). Particularly noticeable are the increased levels of octadecenoic acid (18:1) in the phospholipids. These vary from 2 to 10 times the levels observed in normal liver, depending on the cell line and phospholipid species. Little is known concerning the source of 18:1 (host or hepatoma) in these cells or the mechanism by which they maintain high levels of this fatty acid. The stearoyl-CoA desaturase system, the obligatory step in the biosynthesis of 18:1 in mammalian tissue, has been examined in a variety of transplantable hepatomas (6-10). With the exception of the Morris hepatoma 7800 (10), all tissues examined show low levels of desaturation activity when compared with normal liver. The low desaturase activity is contrary to an abnormally high level of 18:1, leading to the suggestion that the hepatoma depends heavily on the host for the majority of the 18:1 observed and selectively incorporates 18:1 from the surrounding fluid. The metabolic significance of this in vitro data is difficult to interpret without further in vivo studies. In vivo studies using hepatoma maintained within the host are difficult to carry out because of the variability of the host system and complications caused by host contributions or interferences. We have therefore chosen a hepatoma maintained in tissue culture (HTC) in order to coordinate in vitro observations with in vivo metabolic studies. Tissue culture affords a more manipulatable, better defined environment in which to work.

The Morris hepatoma 7288 may be maintained either by transplantation into the host rat (7288CTC) or in tissue culture (7288C). This tissue, maintained in either environment shows similar lipid profiles, including the high 18:1 levels (11-13). The Morris hepatoma 7288C demonstrates the capacity to form 18:1 from acetate, palmitate or stearate when added to the culture medium (14). Because of the compositional similarity and the evidence of an active stearoyl-CoA desaturase system, we have chosen to examine the stearoyl-CoA desaturase system in the Morris hepatoma 7288CTC (CTC) and 7288C (HTC). Results obtained were compared with data obtained from normal liver and host liver. The stearoyl-CoA desaturase system of the HTC and CTC tissue was studied to evaluate the similarity between this tissue in the 2 separate environments, and to assess the validity of relating further metabolic studies concerning 18:1 metabolism using the 7288C to the host-tumor situation.

MATERIALS AND METHODS

Materials and Animals

Buffalo strain rats were obtained from Simonsen Labs (Gilroy, CA) and maintained on a chow diet (Wayne Lab-Blox, Continental

*To whom correspondence should be addressed: Department of Biochemistry, 420 Henry Hall, University of Wisconsin, Madison, WI 53706.

Grain Co., Chicago, IL). [1-^{14}C] Stearoyl-CoA was purchased from Amersham (Arlington Heights, IL). All other substrates and reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Spectrophotometric assays were performed on a Gilford model 252 spectrophotometer (Gilford Instruments Laboratories Inc., Oberlin, OH).

Growth of Morris Hepatoma 7288C and 7288CTC

Morris hepatoma 7288C cells were grown as monolayers in roller cultures using Swim's 77 minimal essential medium. The medium was supplemented with $NaHCO_3$ (2.2 g l^{-1}), glutamine (292 mg l^{-1}), cystine (12 mg l^{-1}), penicillin (60 mg l^{-1}), phenol red (5 mg l^{-1}), $CaCl_2 \cdot 2H_2O$ (265 mg l^{-1}), fetal calf serum (5% by volume) and calf serum (5% volume). This medium will be designated as 5/5. Cells from 4 roller culture bottles were used for each subcellular preparation. The cells were harvested near confluence, medium was removed, the cells were rinsed once with phosphate-buffered saline (PBS), pH 7.4, and a trypsin solution was added to detach cells. After a 2-3 min incubation at 37 C, 5/5 medium was added to stop the reaction. Cells from the 4 roller cultures were combined and formed into pellets by centrifugation at 70 × *g* for 5 min. Cells were washed twice using PBS at 4 C. The final cellular pellet was used immediately for preparing subcellular fractions.

Morris hepatoma 7288CTC was implanted by bilateral intermuscular injection of a homogenate of tumor tissue into the hind limbs of male Buffalo strain rats. Ca. 15 days after the hepatoma was implanted, the host animals, while still in a fed state were sacrificed by decapitation and bled. Hepatoma tissue was removed with the careful avoidance of any necrotic areas. Host liver was also collected at this time. Both liver and hepatoma were minced and washed with homogenization buffer before homogenization.

Preparation of Microsomes

Microsomes were prepared by one of 2 methods. The first involved homogenization of the washed tissue in 6 vol homogenization buffer (0.25 M sucrose, 0.1 M phosphate, 1 mM EDTA, 1 mM dithiothreitol, pH 7.2) using 3 up-and-down strokes of a Potter-Elvehjem tissue homogenizer. The homogenate was filtered through 4 layers of cheesecloth and centrifuged at 700 × *g* for 10 min. The 700 × *g* supernatant was centrifuged at 15,000 × *g* for 20 min to form pellets of the mitochondria. The 15,000 × *g* supernatant was subjected to 100,000 × *g* for 1.5 hr to form pellets of the microsomes. Microsomes were resuspended in 0.1 M phosphate buffer, pH 7.2, for use in assays. The second method of subcellular preparation involved cell disruption using nitrogen cavitation. A cellular pellet from HTC cells or minced and washed tissue from CTC cells was resuspended in 6 vol homogenization buffer (0.25 M sucrose, 0.1 M phosphate, 0.25 mM $MgSO_4$, 1 mM dithiothreitol, pH 7.2) and placed under nitrogen at 1000 psi for 30 min while stirring in a Parr Bomb apparatus (Parr Instrument Co., Moline, IL). The cells were disrupted by slow release through a small apperature. The homogenate was filtered through 4 layers of cheesecloth and brought to 1 mM EDTA. Microsomes were prepared through differential centrifugation steps described previously. All procedures were carried out 0-4 C. Microsomes prepared by either method were used immediately for assays. The method by which a particular microsomal fraction was prepared will be mentioned in the results section.

Enzyme Assays

Overall, microsomal stearoyl-CoA desaturase was assayed by monitoring the desaturation of [1-^{14}C] stearoyl-CoA (15). Cytochrome b_5 content was determined by measuring the reduced minus oxidized spectra of the microsomes at 424 nm and 409 nm (16). Cytochrome b_5 reductase was measured by the method of Oshino and Sato (17) and cytochrome c reductase was measured by the method of Jones and Wakil (18). Protein concentration was determined by the Biuret (19) or Lowry method (20). The amount of protein was varied in each assay to assure that maximal rates were observed.

RESULTS AND DISCUSSION

The microsomal stearoyl-CoA desaturase system was examined in the Morris hepatoma 7288CTC, grown in the host (CTC), host liver and normal liver (Table 1). In these samples, a Potter-Elvehjem tissue homogenizer was used to disrupt the cells. Overall, stearoyl-CoA desaturase activity was reduced by ca. 50% in host liver ($0.10 > P > 0.5$) compared with normal liver. The electron transport system in host liver was also affected, with the cytochrome b_5 content and cytochrome b_5 reductase activity reduced to 71% and 56% of control values. The ability of this system to reduce an artificial electron acceptor, cytochrome c, was also impaired. Microsomal cytochrome c reductase activity in the host liver was reduced to 57% of normal liver. These decreases are probably caused by anorexia in the host animals. In

TABLE 1

In Vitro Analysis of the Stearoyl-CoA Desaturase System in Normal and Host Liver and Morris Hepatoma 7288CTC

	18:0 → 18:1 (pmol min^{-1} mg^{-1})	Cyt b_5 content (pmol mg^{-1})	Cyt b_5 reductase (μmol min^{-1} mg^{-1})	Cyt c reductase (nmol min^{-1} mg^{-1})
Normal liver[a,c]	690 ± 322	433 ± 43	4.74 ± 0.63	995 ± 190
Host liver[b]	346 ± 259	316 ± 34[d]	2.66 ± 0.33[d]	571 ± 126[d]
7288CTC	21 ± 22[d]	9 ± 5[d]	0.69 ± 0.13[d]	82 ± 32[d]

[a]Microsomes prepared with a Potter Elvehjem tissue homogenizer for cell disruption in all tissues.
[b]Host liver taken at the time of tumor tissue harvest.
[c]All values represent the mean ± SD of 4 samples.
[d]$P<0.05$ compared with normal liver values as calculated by the Student's t test.

order to obtain sufficient hepatoma tissue for microsomal preparations (1-2 g), the tissue must be harvested at least 2 weeks after the tumor is implanted. Anorexia is a commonly observed phenomenon in tumor-bearing animals (21) and manifests itself ca. 2-3 weeks after tumors are implanted in this model system. Considering the sensitivity of the stearoyl-CoA desaturase system to starvation (17), decreased activity in the host system is not suprising.

Morris hepatoma microsomes showed extremely low levels of overall activity, only 3% of that observed in normal liver. Examination of the hepatoma electron transport system revealed extreme deficiencies in function. Particularly noticeable were the extremely low levels of cytochrome b_5. Hepatoma microsomes contain 2% of the cytochrome b_5 of normal liver and only 3% of host liver values. Cytochrome b_5 reductase activity was 15% and cytochrome c reductase activity was only 8% of normal liver activity. Increases or decreases in the stearoyl-CoA desaturase activity in liver have been demonstrated to be a direct result of increases or decreases in the level of the terminal desaturase enzyme (22). The electron transport components are less severely affected and are not limiting. Examination of the electron transport system in the Morris hepatoma 7288CTC shows that, not only is overall activity drastically reduced, but the functioning of the electron transport system is deficient. This aberration has been observed in other hepatoma tissues (5,8). The Novikoff hepatoma, which demonstrates no stearoyl-CoA desaturase activity, contains no microsomal cytochrome b_5 (8). Readdition of either purified cytochrome b_5 or terminal enzyme, alone, to the Novikoff hepatoma microsomes had no effect, whereas adding the 2 proteins together restored desaturase activity. Apparently the lack of desaturase activity in this tumor tissue was a result of a deficiency in both the terminal enzyme and cytochrome b_5.

Whether this is the case with the hepatoma tissues used in this study is not known. Flux through the electron transport system appears to be adequate. Cytochrome c reductase activity in these microsomes, a direct function of the cytochrome b_5–cytochrome b_5 reductase complex is reduced in these tissues, but still functions in the nmol min^{-1} mg^{-1} range. The turnover rate of this electron transport complex should be adequate to handle the pmol min^{-1} mg^{-1} rate of the $\Delta 9$ desaturase system. Cytochrome b_5 is used in several other reactions in the cell (23,24) and therefore the terminal desaturase may not be in functional contact with cytochrome b_5 because of competition by other proteins normally associated with this protein.

The microsomal stearoyl-CoA desaturase system in the Morris hepatoma 7288C (HTC), maintained in culture, and Morris hepatoma 7288CTC (CTC) maintained in the host animal (Table 2) were compared. Cells used for microsome preparation were disrupted by nitrogen cavitation. Comparison of the 2 tissues showed that they were similar in their desaturase systems. Little difference was found in overall specific activity of the desaturase system between the CTC and HTC tissue. HTC microsomes showed a higher overall specific activity but the activity was still extremely low when compared with normal liver values. Cytochrome b_5 content and cytochrome b_5 reductase activities were similar, but cytochrome c reductase activity was 3-to-4 fold higher in the HTC microsomes than in the CTC microsomes. This discrepancy between the 2 tissues is, as yet, unexplained. Microsomes from CTC tissue, prepared using the nitrogen cavitation method, showed higher levels of cytochrome b_5 reductase activity compared with that of microsomes prepared with the Potter-Elvehjem tissue homogenizer (Table 1). The reason for this is also unclear. Normal liver microsomes prepared by either method showed identical values in all

TABLE 2

In Vitro Analysis of the Stearoyl-CoA Desaturase System in Morris Hepatoma 7288C and 7288CTC

	18:0 → 18:1 (pmol min^{-1} mg^{-1})	Cyt b_5 content (pmol mg^{-1})	Cyt b_5 reductase (μmol min^{-1} mg^{-1})	Cyt c reductase (nmol min^{-1} mg^{-1})
7288CTC[a,b]	47 ± 8	10 ± 2	1.92 ± 1.25	69 ± 19
7288C[a,c]	81 ± 28	10 ± 5	2.36 ± 0.57	267 ± 64[d]

[a]Microsomes prepared using nitrogen cavitation for cell disruption.
[b]All values represent the mean ± SD of 4 samples.
[c]Morris hepatoma 7288C cells were grown in roller culture in Swin's 77 medium supplemented with 5% fetal calf serum and 5% bovine serum (5/5 medium). Cells were harvested before confluence.
[d]$P<0.05$ compared with values for Morris hepatoma 7288CTC as determined by the Student's t test.

4 parameters measured (data not shown).

The Morris hepatoma 7288C and 7288CTC, tissues with relatively high levels of 18:1, showed decreased stearoyl-CoA desaturase activity when compared with either host or normal liver. These results agree with data obtained from other hepatoma tissues (6-9). The metabolic significance of these data is, as yet, unknown. Several explanations may justify low desaturase activity, measured in vitro, with high 18:1 levels. The stearoyl-CoA desaturase system in hepatoma tissue may be more sensitive to mechanical manipulation and therefore lower activity results. This is not unreasonable because of the abnormal composition of the membranes in this tissue (25). Researchers have suggested that these cells may obtain most, if not all, of the 18:1 from the surrounding milieu. Watson (26) has demonstrated that the Morris hepatoma 7288C in spinner culture makes only 15-20% of the fatty acids required during growth, through de novo synthesis. This finding supports the 18:1 being of primarily exogenous origin. These calculations, however, do not take into account the 18:1 that is produced through desaturation of exogenous long-chain fatty acids; a route that is dependent, also, on the stearoyl-CoA desaturase system. The importance of the stearoyl-CoA desaturase system for the maintenance of high 18:1 levels in these cells, therefore, remains unresolved. Further in vivo studies are underway in our laboratory to answer this question, using the Morris hepatoma 7288C. The results obtained from these studies will be related to the in vitro data obtained in both the Morris hepatoma 7288C and 7288CTC.

REFERENCES

1. Howard, B., Butler, J.D., and Bailey, J.M. (1972) in Tumor Lipids: Biochemistry and Metabolism (Wood, R., ed.) pp. 200-214, American Oil Chemists' Soceity, Champaign IL.
2. Van Hoeven, R.P., and Emmelot, P. (1972) in Tumor Lipids: Biochemistry and Metabolism (Wood, R., ed.) pp. 126-138, American Oil Chemists' Society, Champaign, IL.
3. Waite, M., Parce, B., Morton, R., Cunningham, C., and Morris, H.P. (1977) Cancer Res. 37, 2902-2908.
4. Reitz, R.C., Thompson, J.A., and Morris, H.P. (1977) Cancer Res. 37, 561-567.
5. Morton, R., Cunningham, C., Jester, R., Waite, M., Miller, N., and Morris, H.P. (1976) Cancer Res. 36, 3246-3254.
6. Morton, R.E., Hartz, J.W., Reitz, R.C., Waite, M., and Morris, H.P. (1979) Biochim. Biophys. Acta 573, 321-331.
7. Lee, T., Stephens, N., and Snyder, F. (1974) Cancer Res. 34, 3270-3273.
8. Prasad, M.R., and Joshi, V.C. (1979) Lipids 14, 413-415.
9. Chiappe, L., Mercuri, O., and de Thomas, M.E. (1974) Lipids 9, 489-490.
10. Raju, P.K. (1974) Lipids 9, 795-797.
11. Wood, R. (1973) Lipids 8, 690-701.
12. Wood, R., Falch, J., and Wiegand, R.D. (1975) Lipids 10, 202-207.
13. Wood, R. (1975) Lipids 10, 736-745.
14. Weigand, R., and Wood, R. (1975) Lipids 10, 194-201.
15. Jones, P.D., Holloway, P.W., Peluffo, R.O., and Wakil, S.J. (1969) J. Biol. Chem. 249, 744-754.
16. Omura, J., and Sato, R. (1964) J. Biol. Chem. 239, 2370-2378.
17. Oshino, N., and Sato, R. (1972) Arch. Biochem. Biophys. 149, 369-377.
18. Jones, P.D., and Wakil, S.J. (1967) J. Biol. Chem. 242, 5267-5273.
19. Cornall, A.G., Bardawill, G.J., and David, M.M (1949) J. Biol. Chem. 177, 751-755.
20. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
21. DeWys, W. (1970) Cancer Res. 30, 2816-2818.
22. Joshi, V.C., and Aranda, L.P. (1979) J. Biol. Chem. 254, 11779-11782.
23. Shenkman, J.B., Jansson, I., and Robie-Suh, K.M. (1976) Life Sciences 19, 611-624.
24. Keyes, S.R., Alfano, J.A., Jansson, I., and Cinti, D.L. (1979) J. Biol. Chem. 254, 7778-7784.
25. Upreti, G., de Antueno, R.J., and Wood, R.D. (1983) J. Natl. Canc. Inst. 70, 567-573.
26. Watson, J.A. (1972) in Tumor Lipids: Biochemistry and Metabolism (Wood, R., ed.) p. 34-53, American Oil Chemists' Society, Champaign, IL.

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Analyses of Lipids and Fatty Acids in Ripe Roes of Some Northwest European Marine Fish

DOUGLAS R. TOCHER* and **JOHN R. SARGENT**, *Natural Environment Research Council, Institute of Marine Biochemistry, Aberdeen, AB1 3RA, United Kingdom*

ABSTRACT

Lipid class analyses and fatty acid analyses of neutral and polar lipids were carried out on ripe roes of herring, cod, haddock, whiting, saithe, sand eel and capelin. Total lipid was 10-26% of roe dry weight. The species with the highest total lipid, sand eel and capelin, also had the highest percentage of neutral lipid in total lipid, 77% and 49% respectively. In the other species, phospholipids accounted for 62-77% of roe total lipid. Both the neutral lipids, and especially the phospholipids, of all species were very unsaturated because of high concentrations of (n-3) polyunsaturated fatty acids (PUFA), frequently amounting to 50% of the total egg lipid. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) had similar fatty acid compositions in all species, with an average ratio (n-3)/(n-6) of ca. 20:1. Phosphatidylinositol (PI) consistently had high concentrations of 18:0 and 20:4 (n-6) with an average ratio of (n-3)/(n-6) of 1.8:1. Requirements for high levels of (n-3) PUFA in the embryonic and early larval development stages of marine fish are suggested as is a special role for the 20:4(n-6) in PI.

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INTRODUCTION

Marine lipids contain high concentrations of PUFA with an (n-3)/(n-6) ratio in the region of 10-15:1 (1). The origins of (n-3) PUFA in the marine phytoplankton and their importance in the marine food web, particularly as essential dietary factors for marine fish, are well known (2), and indications have been found that (n-6) PUFA may also be essential dietary factors in marine fish (3,4).

Much research, relevant to commercial marine fisheries and aquaculture alike, is currently being directed toward understanding factors influencing the survival and growth of larval fish. These fish appear to have rather exacting requirements for dietary PUFA and methods have been developed for providing them with relatively high concentrations of marine PUFA in both natural and synthetic diets (5,6).

Fish eggs contain supplies of PUFA that are optimized nutritionally for growth of the embryo and also for growth of the resultant larva up to the stage of yolk sac absorption. Numerous analyses of the lipids of fish eggs have been published and, although the literature is fragmentary, PUFA clearly can account for more than 40% of the total fatty acids in marine fish eggs, reflecting at least in part, the high concentrations of phospholipids in the egg (7-9). However, few if any studies have been made of fish egg lipids where complete lipid class analyses together with fatty acid analyses of individual neutral lipids and phospholipids have been presented. Recently we reported that PI in the salt-secreting epithelia of 2 marine fish was unusual in containing relatively high concentrations of arachidonic acid and stearic acid and we discussed the implications of this finding in terms of fish nutrition (4). The present study stems from that result and is concerned with defining the distribution of (n-3) and (n-6) PUFA in individual lipid classes in fish eggs so as to elucidate the role of these fatty acids in embryonic and subsequent larval development.

*To whom correspondence should be addressed.

MATERIALS AND METHODS

Fish Eggs

Ripe roe was excised from the following fish: cod (*Gadus morhua*) caught during March 1983 off Gourdon, Scotland; Atlantic herring (*Clupea harengus*), haddock (*Melanogrammus aeglefinus*), saithe (*Pollachius virens*) and whiting (*Merlangus merlangus*) caught at the end of March 1983 on the Ballantrae Bank in the lower Clyde estuary, Scotland; sand eel (*Ammodytes lancea*) (10) taken in Loch Ewe, Scotland, during the summer of 1983; capelin (*Mallotus villosus*) taken in Balsfjorden, northern Norway, in May 1981. All roe samples were stored at -80 C before they were analyzed.

Analyses

Moisture contents were determined by freeze-drying replicate samples in an Edwards "Speedivac" centrifugal freeze dryer. Total lipid was extracted from freeze-dried eggs by

the method of Folch et al. (1957) (11) and stored at -20 C between analytical procedures. Polar and neutral lipids were separated by thin layer chromatography (TLC) on silica gel 60 plates using hexane/diethyl ether/acetic acid (80:20:2 by vol) as solvent. Neutral lipids were eluted with chloroform/methanol (2:1); polar lipids with chloroform/methanol/water (5:5:1). The eluted lipids were dried under N_2 and relative proportions determined gravimetrically. For quantitative analyses the neutral lipids were separated by TLC using hexane/diethyl ether/acetic acid (80:20:1) after which the plates were stained with copper acetate/phosphoric acid reagent (12) and scanned in a Vitatron TLD 100 densitometer coupled to a Hewlett Packard 3380A recording integrator. Polar lipids were separated using 2-dimensional TLC (13) and visualized by brief and minimal exposure to iodine vapor; eluted and dried phospholipids were quantified by the colorimetric estimation of phospholipid-bound phosphorus (14).

Procedures similar to the above were used to separate individual neutral lipids and phospholipids for fatty acid analyses, except that all solvents were supplemented with 0.05% butylated hydroxytoluene. Methyl esters of fatty acids were prepared by acid-catalyzed transmethylation of individual lipids (15), then purified by TLC. The putative steryl ester zones in neutral lipid analyses, when transmethylated and purified by TLC, yielded fatty acid methyl esters, free sterol and only traces of free fatty alcohols. Fatty acid methyl esters were analyzed in a Packard 429 gas chromatograph (GC) (Packard Instruments Inc., Caversham, U.K.) equipped with a CP Wax 51 fused capillary column (50 m × 0.34 mm i.d.) (Chrompack, U.K. Ltd., London) using helium as carrier gas and a thermal gradient from 140 C to 225 C. Individual methyl esters were identified by comparison with known standards and also by reference to published data (1,16) and quantified using a Hewlett-Packard 3390A recording integrator.

RESULTS AND DISCUSSION

All analyses were carried out on the roes of ripe female fish immediately before spawning. At this time the bulk of ovary consists of eggs rather than ovarian tissue, so that the results here can reasonably be taken to represent analyses of the eggs themselves. In all species, except capelin, the roes were taken from samples of fish, some individuals of which were separately stripped and the released eggs successfully fertilized by the addition of milt. The capelin population sampled was observed spawning spontaneously less than 1 hr after the roe samples were taken.

The total lipid contents of the ripe ovaries studied varied from ca. 10% to over 26% of the dry weight in the different species investigated (Table 1). In the species with total lipid contents of 10-15% the majority of the lipid (70% on average) was polar lipid. Sand eel and capelin eggs had higher lipid contents than the other species and also contained more neutral lipid, over 75% in the case of sand eel eggs, which are known to have distinct oil globules. The eggs of cod, saithe, haddock and whiting are planktonic whereas herring eggs, with a similar lipid content, are spawned on the bottom and adhere to the stratum (17). Furthermore, the eggs with the highest total and neutral lipid contents, capelin and sand eel, and therefore, presumably, the most positively bouyant are not planktonic but are spawned on the shore or on the bottom (17). This suggests that although the total and neutral lipid contents may affect buoyancy, they do not have a functional role in this respect, and that other factors such as area of spawning, currents, tides and egg-specific features have a more important role. However, both the total and neutral lipid contents of the eggs correlate with the time interval between fertilization and hatching. Sand eel eggs have an incubation time of ca. 27 days, capelin eggs have an incubation time of ca. 23 days and the other species all have incubation times of 10-16 days, all at 8 C (17, 18). This is in line with the well-known role of neutral lipids as energy reserves in embryonic and early larval development in fish (19-21).

Triacylglycerols were generally the major neutral lipid class with free sterol also being notable. However, in capelin and sand eel eggs the concentration of steryl esters exceeded that of free sterols. The steryl/wax ester zone after transmethylation and TLC analyses indicated only traces of wax esters in all the species examined. Whiting eggs contained a relatively high concentration of free fatty acids, which may be a decomposition artefact, although this was not reflected in the values for partial acyl glycerols or lysophosphatides in this species. Of the polar lipids, PC and PE generally constituted ca. 70% and 20% of the total polar lipids, respectively, with PI accounting for 2.5-4.5%.

The fatty acid composition of the polar lipid fractions show, on average, 29% saturated fatty acids (primarily 16:0), 19% monoenes (primarily 18:1 isomers) and almost 50% PUFA, of which 94% was (n-3) isomers (mainly 20:5 and 22:6), giving an (n-3)/(n-6) ratio of 14.6 (Table 2). Variation in the (n-3)/(n-6) ratio between the

TABLE 1

Lipid Contents and Lipid Class Analysis of Roes of Various Fish Species

	Cod[b]	Herring[b]	Haddock[b]	Whiting[b]	Saithe[a]	Capelin[c]	Sand eel[c]
Egg diameters (mm)	1.3–1.4	1.3–1.5	1.2–1.4	1.0–1.1	0.9–1.1	1.0–1.2	0.3
Moisture content (%)[d]	74	74	86	85	72	70	64
Lipid content[d] (% dry wt)	13.2 ± 0.4	14.6 ± 0.5	10.7 ± 0.2	10.6 ± 1.0	15.4 ± 0.3	26.3 ± 3.6	19.7 ± 0.9
Polar lipids (%)[d]	71.7 ± 1.0	69.0 ± 0.6	71.3 ± 7.7	61.2 ± 0.6	66.5 ± 1.7	50.7 ± 1.6	23.4 ± 4.6
Neutral lipids (%)[d]	28.3 ± 1.0	31.0 ± 0.6	28.7 ± 7.7	38.8 ± 0.6	33.5 ± 1.7	49.3 ± 1.6	76.6 ± 4.6
Neutral classes[e] (% total lipid)							
Triacylglycerol	12.5 ± 0.4	14.8 ± 0.5	8.3 ± 0.7	7.3 ± 0.5	14.3 ± 0.7	30.4 ± 0.4	45.7 ± 0.8
Cholesterol	6.1 ± 0.3	8.3 ± 0.9	9.5 ± 0.1	11.7 ± 0.5	11.1 ± 0.7	3.1 ± 0.1	4.7 ± 0.5
Free fatty acids	5.4 ± 0.3	5.7 ± 0.3	5.3 ± 0.3	13.2 ± 0.2	4.5 ± 0.3	6.9 ± 0.2	1.5 ± 0.5
Sterol esters	3.7 ± 0.7	1.0 ± 0.2	3.9 ± 0.3	4.4 ± 0.7	1.7 ± 0.3	7.3 ± 0.2	22.8 ± 0.9
Diacylglycerols	0.6 ± 0.1	0.4 ± 0.2	0.6 ± 0.1	1.1 ± 0.2	1.0 ± 0.2	1.1 ± 0.1	1.8 ± 0.4
Monoacylglycerols	N.D.	0.7 ± 0.1	1.0 ± 0.2	1.1 ± 0.1	1.0 ± 0.4	N.D.	N.D.
Fatty alcohols	trace	0.3 ± 0.1	trace	N.D.	N.D.	N.D.	0.5 ± 0.1
Wax esters	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Polar classes[d] (% total lipid)							
Phosphatidylcholine	45.6 ± 1.2	57.6 ± 0.8	45.8 ± 1.3	40.4 ± 1.7	49.6 ± 0.4	37.7 ± 0.9	16.7 ± 0.8
Phosphatidylethanolamine	20.2 ± 0.4	6.7 ± 0.9	15.4 ± 1.7	13.9 ± 1.2	12.3 ± 0.1	7.6 ± 0.1	7.6 ± 0.3
Phosphatidylinositol	3.0 ± 0.4	1.4 ± 0.2	3.2 ± 0.5	2.1 ± 0.1	1.7 ± 0.1	2.0 ± 0.1	1.1 ± 0.1
Phosphatidylserine	1.4 ± 0.1	0.7 ± 0.3	3.2 ± 0.2	1.6 ± 0.3	1.2 ± 0.2	1.0 ± 0.1	0.9 ± 0.3
Sphingomyelin	0.4 ± 0.4	0.6 ± 0.1	1.3 ± 0.1	1.5 ± 0.1	1.1 ± 0.1	1.4 ± 0.5	0.8 ± 0.1
Lyso-PC	0.9 ± 0.1	1.9 ± 0.2	2.0 ± 0.1	1.5 ± 0.2	0.6 ± 0.2	1.0 ± 0.3	1.4 ± 0.2
Phosphatidic acid	0.5 ± 0.4	trace	0.5 ± 0.1	0.2 ± 0.1	N.D.	N.D.	0.5 ± 0.2

Samples of eggs were pooled from the roe of a, 2; b, 3 and c, 6 fish. Results are means ± SD of d, 3 and 3, 10 analyses. N.D. = not detected; trace = < 0.2%.

TABLE 2

Fatty Acid Composition of Total Phospholipids from Roes[a]

	Cod	Herring	Saithe	Haddock	Whiting	Capelin	Sand eel
14:0	1.5	1.8	1.1	1.2	0.9	2.8	1.8
16:0	23.7	27.4	23.1	21.4	25.3	22.1	23.9
16:1(n-9)	0.9	0.5	0.8	0.8	1.1	0.4	0.4
16:1(n-7)	2.6	2.9	2.4	2.6	1.7	3.1	2.9
17:0	0.3	0.3	0.4	0.5	0.4	0.4	0.3
18:0	2.3	2.7	3.5	2.4	3.3	2.1	2.9
18:1(n-9)	11.0	4.8	13.6	8.3	11.0	8.2	7.0
18:1(n-7)	3.6	5.8	4.1	7.0	4.1	3.4	2.0
18:2(n-6)	1.1	0.5	1.3	0.7	0.7	0.8	1.7
18:3(n-3)	0.3	0.3	0.6	0.3	0.1	0.6	0.7
18:4(n-3)	0.3	0.3	0.5	–	0.1	0.6	0.8
20:1[b]	2.1	0.5	2.3	1.4	1.5	2.0	1.4
20:4(n-6)	1.9	1.0	1.6	3.7	2.4	1.1	1.9
20:4(n-3)	0.4	0.3	0.7	0.3	0.3	0.6	0.6
20:5(n-3)	15.3	13.7	11.5	12.6	13.3	19.0	16.7
22:1	–	–	–	–	–	–	1.4
22:5(n-3)	1.2	1.0	1.1	3.2	1.2	1.7	1.0
22:6(n-3)	28.6	31.4	27.7	27.6	30.3	24.6	25.5
24:1	0.1	–	0.1	0.3	0.1	1.0	0.7
Total saturates[c]	28.1	32.7	28.1	25.5	29.9	27.4	29.1
Total monoenes	20.3	14.5	23.3	20.4	19.5	18.1	15.8
Total (n-3)[c]	46.1	47.1	42.3	44.3	45.3	47.1	45.3
Total (n-6)[c]	3.0	1.6	3.1	5.1	3.1	1.9	3.9
(n-3)/(n-6)	15.4	29.4	13.7	8.7	14.6	24.8	11.6
Total unknowns	2.6	4.1	3.3	4.8	2.4	4.8	6.1

[a]Composition as percentage of weight. Values are means of duplicate or triplicate analyses. Standard deviations are omitted for clarity but were normally <5%.
[b]Predominantly 20:1(n-9).
[c]Totals include 20:2(n-6); 22:0, 22:4 isomers and 24:0, present in some samples at <0.5%.

different species stemmed mainly from variation in the (n-6) levels. The neutral lipid fraction contained on average ca. 24% saturates, again mainly the 16:0 (Table 3) found in the polar fraction (Table 2). However, the percentage of PUFA in the neutral lipid fraction was reduced to 37% with a corresponding increase in the percentage of monoenes, mainly 16:1 and 18:1 isomers, to 35% (Table 3). The (n-3)/(n-6) ratio in the neutral lipid fractions averaged 10:6.

The fatty acid compositions of the polar lipid fractions were generally similar in all species (Table 2), the same holding true for the neutral lipid fractions (Table 3), suggesting that the diet of the parent fish has relatively little effect in determining the final fatty acid composition of egg lipids, a conclusion reached earlier (7). However, haddock eggs had higher concentrations of (n-6) fatty acids than the other species and differences do occur in the percentages of individual fatty acids in the other species. Clearly some influence of parental diet on egg-lipid composition occurs. In the present study we determined the fatty acid compositions of the total roe lipid in the different species. The data for total lipid (not shown) were similar to those for polar lipid in all species except capelin and sand eel where the higher levels of neutral lipid in the roes generate somewhat lower percentages of PUFA in total roe lipid.

PI and its phosphorylated derivatives, although quantitatively minor components of biomembranes, play critical roles in regulating numerous major metabolic processes (22). Researchers had shown earlier that PI from garfish olfactory nerve (23), trout liver (24) and trout total body lipid (25) contains a relatively high percentage of saturates, mainly 18:0, and a low (n-3)/(n-6) ratio stemming from a high concentration of 20:4(n-6). This pattern, similar to that of a terrestrial mammalian phospholipid, has recently also been found in plasma membrane fractions from cod gills and dogfish rectal gland (4). Bell et al. have proposed (4) that the consistent composition of PI results from this phospholipid playing the same unique biomembrane role in fish as it does in terrestrial mammals. Table 4 shows that the PI from all the species studied contains relatively high concentrations of (n-6) PUFA, mainly 20:4(n-6), giving, on average, a low

TABLE 3

Fatty Acid Composition of Total Neutral Lipid from Roes[a]

	Cod	Herring	Saithe	Haddock	Whiting	Capelin	Sand eel
14:0	3.3	3.6	2.8	2.5	1.3	7.1	4.9
16:0	15.0	24.6	13.2	15.0	17.4	16.1	19.4
16:1(n-9)	0.7	0.7	0.6	1.0	1.7	0.3	0.5
16:1(n-7)	6.8	7.0	5.6	7.9	3.5	9.2	8.7
17:0	0.7	0.6	0.5	0.6	0.5	0.5	0.3
18:0	2.3	1.8	3.4	2.3	2.3	1.2	2.1
18:1(n-9)	18.2	18.3	21.2	11.3	17.3	13.4	8.1
18:1(n-7)	5.0	5.6	4.9	8.0	4.8	3.8	2.4
18:2(n-6)	2.0	1.5	1.9	1.0	0.9	2.1	2.4
18:3(n-3)	1.0	1.5	1.5	0.8	0.5	1.7	1.7
18:4(n-3)	1.3	1.1	2.2	1.2	0.6	2.0	2.0
20:1[b]	5.7	1.4	5.7	3.2	2.2	6.5	3.4
20:4(n-6)	1.2	0.6	1.2	2.8	2.4	0.5	0.9
20:4(n-3)	0.8	0.6	1.1	0.7	0.6	0.8	1.5
20:5(n-3)	10.9	9.7	9.7	14.0	15.0	13.1	12.4
22:1	3.6	0.5	2.1	0.9	0.8	2.7	2.7
22:5(n-3)	1.5	0.8	1.6	2.9	1.4	0.9	1.4
22:6(n-3)	16.0	17.1	14.8	14.5	22.3	14.1	18.8
24:1	1.5	–	1.4	0.6	0.9	–	0.1
Total saturates	21.3	30.6	19.9	20.4	21.5	24.9	26.7
Total monoenes	41.5	33.5	41.5	32.9	31.2	35.9	25.9
Total (n-3)[c]	31.5	30.8	30.9	34.4	40.4	32.6	38.0
Total (n-6)[c]	3.2	2.1	3.4	4.2	3.3	2.6	3.6
(n-3)/(n-6)	9.8	14.7	9.1	8.2	12.2	12.5	10.6
Total unknowns	2.8	2.8	4.3	6.4	2.7	4.0	5.9

[a]Composition as percentage of weight. Values are means of duplicate or triplicate analyses. Standard deviations are omitted for clarity but were normally <5%.

[b]Predominantly 20:1(n-9).

[c]Totals include 20:2(n-6) and 22:4 isomers present in some samples at <0.5%.

(n-3)/(n-6) ratio of 1.8. A relatively high concentration of 18:0 was also observed, although the total percentages of saturates and monounsaturates in PI were similar to those in total polar lipid. The major phospholipids in roes, PC and PE had fatty acid compositions similar to that of the total polar lipid fraction, with high (n-3)/(n-6) ratios, which again showed some variation depending on the level of (n-6) (Table 5 and 6). PE was the only phospholipid containing plasmalogens as shown by the presence of dimethylacetals after acid-catalyzed transmethylation but the amounts present were generally low (Table 6).

In general, the data here provide an insight into the lipid and fatty acid requirements of the developing embryos and early larvae of marine fish, because the egg is a repository of all the essential nutrients required for development of the fertilized egg up to the larval feeding stage. The generally comparable fatty acid composition of egg lipids (whether total, polar or individual classes) between the species studied points to involvement of these lipids in basic functions common to all the species. The consistently high concentrations of PUFA, generally approaching 50% of the total fatty acids in polar and indeed total lipids, is particularly noteworthy. The PUFA content of the eggs is ca. 5-10% of the egg dry weight and because the chorion contributes significantly to the dry weight of the egg, the PUFA content of the egg yolk itself must be even higher. In a sense, the development of fertilized eggs and larvae up to the stage of yolk sac absorption requires nutrients with a very high content of (n-3) PUFA. Whether such a requirement extends beyond the stage of yolk sac absorption is not known but the implications of such a possibility in early larval nutrition are obvious. The reasons underlying the very high content of (n-3) PUFA in fish eggs are not currently known. One possibility is that phospholipids are more easily mobilized than triacylglycerols in eggs so that the former lipid is the preferred energy reserve and a high concentration of (n-3) PUFA is an inevitable consequence. This proposition is not easily reconciled with the observation here that eggs with longer development times, i.e., containing larger energy reserves, are richer in triacylglycerols than phospholipids. An alternative pos-

TABLE 4

Fatty Acid Composition of Phosphatidylinositol (PI) from Roes[a]

	Cod	Herring	Saithe	Haddock	Whiting	Capelin	Sand eel
14:0	4.1	9.1	7.0	0.7	0.2	10.9	1.1
15:0	2.4	1.6	3.1	0.5	0.9	2.8	0.6
16:0	13.8	17.0	13.9	10.6	12.4	17.8	13.1
16:1(n-9)	2.4	2.0	0.8	0.7	0.7	1.1	0.7
16:1(n-7)	1.3	1.9	0.4	1.0	0.5	0.4	1.3
17:0	0.5	0.4	0.5	0.5	0.7	0.6	0.4
18:0	12.1	14.3	15.3	17.1	15.9	14.2	14.9
18:1(n-9)	10.0	7.9	10.9	8.1	10.3	7.8	7.3
18:1(n-7)	2.4	3.9	2.2	6.8	4.7	1.8	2.9
18:2(n-6)	0.7	0.8	0.7	0.5	0.3	0.7	0.6
18:3(n-6)	–	–	–	–	0.5	0.5	1.3
20:0	–	–	–	0.7	–	–	–
20:1[b]	1.5	0.9	3.0	2.2	3.9	3.3	1.3
20:4(n-6)	8.1	4.9	12.9	18.5	10.5	5.3	9.2
20:4(n-3)	0.3	0.6	–	–	0.6	–	0.9
20:5(n-3)	9.2	11.5	5.8	10.1	9.5	8.3	14.1
22:5(n-3)	0.8	0.4	0.3	1.9	0.6	0.5	0.9
22:6(n-3)	10.9	7.4	7.7	10.2	12.3	3.6	9.6
24:1	0.9	–	1.1	–	0.6	1.9	1.3
Total saturates	32.9	42.5	39.8	30.1	30.1	46.3	30.1
Total monoenes	18.5	16.6	18.4	18.8	20.7	16.3	14.8
Total (n-3)	21.2	19.9	13.8	22.2	23.0	12.4	25.5
Total (n-6)	8.8	5.7	13.6	19.0	11.3	6.5	11.1
(n-3)/(n-6)	2.4	3.5	1.0	1.2	2.0	1.9	2.3
Total unknowns	13.4	15.2	14.6	10.2	14.6	19.3	15.7

[a]Composition as percentage of weight. Values are means of duplicate or triplicate analyses. Standard deviations are omitted for clarity but were normally <5%.
[b]Predominantly 20:1(n-9).

TABLE 5

Fatty Acid Composition of Phosphatidylcholine (PC) from Roes[a]

	Cod	Herring	Saithe	Haddock	Whiting	Capelin	Sand eel
14:0	2.2	2.5	2.0	1.4	0.8	4.5	1.5
15:0	0.4	–	0.8	–	–	1.3	–
16:0	28.7	29.2	24.7	26.2	26.5	22.7	23.3
16:1(n-9)	1.3	0.6	0.9	1.1	1.3	1.1	0.4
16:1(n-7)	3.1	3.6	2.4	3.2	1.6	3.9	2.8
18:0	1.9	1.8	3.2	1.3	2.0	1.4	2.0
18:1(n-9)	11.1	5.5	13.3	8.5	11.0	7.1	7.1
18:1(n-7)	2.8	5.4	3.4	5.7	3.2	2.4	2.1
18:2(n-6)	0.9	0.7	1.1	0.5	0.5	0.8	1.5
18:3(n-3)	0.3	0.4	0.5	0.2	–	0.6	0.6
18:4(n-3)	0.3	0.6	0.7	–	–	0.6	0.8
20:1[b]	1.9	0.3	2.2	0.8	1.5	1.9	1.3
20:4(n-6)	1.5	0.6	1.0	3.0	1.9	0.5	1.5
20:4(n-3)	0.3	0.3	0.6	0.3	0.3	0.6	0.6
20:5(n-3)	13.2	15.9	11.4	14.0	14.7	18.3	17.8
22:1	–	–	–	–	–	–	1.0
22:5(n-3)	1.1	0.8	1.0	2.6	1.1	1.3	1.1
22:6(n-3)	24.7	26.5	25.8	21.2	26.7	21.4	29.4
Total saturates[c]	33.1	33.8	31.4	28.9	29.3	30.3	28.2
Total monoenes	20.2	15.4	22.2	19.3	19.0	16.4	13.7
Total (n-3)[c]	39.9	44.5	40.2	38.3	42.8	42.8	50.3
Total (n-6)[c]	2.4	1.5	2.5	3.5	2.4	1.3	3.0
(n-3)/(n-6)	16.6	29.7	16.1	10.9	17.8	32.9	16.8
Total unknowns	4.0	4.8	3.5	8.5	4.3	4.8	4.3

[a]Composition as percentage of weight. Values are means of duplicate or triplicate analyses. Standard deviations are omitted for clarity but were normally <5%.

[b]Predominantly 20:1(n-9).

[c]Totals include 17:0, 18:3(n-6), 20:2(n-6), 20:3(n-6), 22:0, 22:4 isomers and 24:0 present in some samples at <0.5%.

sibility, currently under investigation, is that the phospholipids in egg are destined mainly for biomembranes in the developing organism and that the high initial concentration of these lipids in egg yolk is related to the rapid proliferation of cells and new biomembranes in the developing organism. Finally, despite the emphasis on (n-3) PUFA in egg lipids, an important and possibly essential role for 20:4 (n-6) in development, previously postulated for marine salt secreting tissues (4), is indicated by the unique and consistent fatty acid composition of PI, even at the earliest stage of the fishes' life history.

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REFERENCES

1. Ackman, R.G. (1980) in Advances in Fish Science and Technology (Connell, J.J., ed.), pp. 86-103, Fishing News Book Ltd., Farnham, Surrey, U.K.
2. Sargent, J.R., and Whittle, K.J. (1981) in Analysis of Marine Ecosystems (Longhurst, A.R., ed.) pp. 491-533, Academic Press, London.
3. Watanabe, T. (1982), Comp. Biochem. Physiol. 73B, 3-15.
4. Bell, M.V., Simpson, C.M.F., and Sargent, J.R. (1983) Lipids, 18, 720-726.
5. Imada, O., Kageyama, Y., Watanabe, T., Kitajima, C., Fujita, S., and Yone, Y. (1979) Bull. Jap. Soc. Sci. Fish. 45, 955-959.
6. Watanabe, T., Tamiya, T., Oka, A., Hirata, M., Kitajima, C., and Fujita, S. (1983) Bull. Jap. Soc. Sci. Fish. 49, 471-479.
7. Lasker, R., and Theilacker, G.H. (1962) J. Lipid Res. 3, 60-64.
8. Ackman, R.G., and Burgher, R.D. (1964) J. Fish. Res. Bd. Canada 21, 469-476.
9. Kaitaranta, J.K., and Ackman, R.G. (1981) Comp. Biochem. Physiol. 69B, 725-729.
10. Wheeler, A. (1969) The Fishes of the British Isles and North-West Europe, MacMillan, London.
11. Folch, J., Lees, M., and Sloane Stanley, G.H. (1957) J. Biol. Chem. 226, 497-509.
12. Fewster, M.E., Burns, B.J., and Mead, J.F. (1969) J. Chromatogr. 43, 120-126.
13. Parsons, J.G., and Patton, S. (1967) J. Lipid Res. 8, 696-698.
14. Raheja, R.K., Kaur, C., Singh, A., and Bhatia, I.S. (1973) J. Lipid Res. 14, 695-697.
15. Christie, W.W. (1973) in Lipid Analyses, Per-

TABLE 6

Fatty Acid Composition of Phosphatidylethanolamine (PE) from Roes[a]

	Cod	Herring	Saithe	Haddock	Whiting	Capelin	Sand eel
14:0	2.4	4.1	2.6	0.8	1.1	5.5	1.5
15:0	0.6	0.8	0.6	0.3	0.4	0.9	–
16:0	16.6	16.8	14.1	13.7	15.7	16.7	12.8
16:1(n-9)	0.8	0.6	0.7	0.5	1.0	–	0.2
16:1(n-7)	1.4	2.2	0.9	1.4	0.9	1.5	1.7
18:0	3.5	4.2	4.5	1.6	2.7	4.2	3.1
18:1(n-9)	11.4	6.1	14.0	8.4	10.2	12.0	6.8
18:1(n-7)	5.5	10.4	5.2	11.5	5.9	4.9	3.2
18:2(n-6)	1.3	0.7	1.3	1.4	1.6	1.3	2.3
18:3(n-3)	0.3	1.1	0.4	0.4	–	–	0.7
18:4(n-3)	–	–	1.5	–	–	1.3	0.4
20:1[b]	3.4	1.3	3.4	2.7	4.3	3.0	2.5
20:2(n-6)	0.4	–	–	0.5	0.5	–	0.5
20:4(n-6)	1.4	0.5	1.0	2.2	1.6	0.8	2.0
20:4(n-3)	0.3	–	0.8	0.5	0.4	–	0.6
20:5(n-3)	14.2	11.1	10.5	10.2	11.0	13.0	13.1
22:1	–	–	–	–	–	–	1.7
22:5(n-3)	1.1	0.8	1.2	3.2	1.3	0.9	1.1
22:6(n-3)	28.7	24.4	30.8	31.3	36.0	17.0	31.0
24:1	–	–	–	–	–	1.8	0.9
Total saturates[c]	23.1	25.9	22.0	16.9	19.9	27.3	17.4
Total monoenes	22.5	20.6	24.2	24.5	22.3	23.2	17.0
Total (n-3)[c]	44.6	37.4	45.4	46.0	48.7	32.2	46.9
Total (n-6)[c]	3.9	1.2	2.3	4.5	3.7	2.1	4.8
(n-3)/(n-6)	11.4	31.2	19.7	10.2	13.1	15.3	9.8
Total dimethylacetals[d]	0.7	0.9	0.5	2.3	3.1	2.7	2.4
Total unknowns	3.0	13.5	5.4	4.5	2.4	13.8	9.6

[a]Composition as percentage of weight. Values are means of duplicate or triplicate analyses. Standard deviations are omitted for clarity but were normally <5%.
[b]Predominantly 20:1(n-9).
[c]Total include 17:0, 20:3(n-6) and 22:4 isomers present in some samples at <0.5.
[d]Dimethylacetals were invariably C16, C17 and C18.

gammon Press, Oxford, England.
16. Ackman, R.G., and Eaton, C.A. (1978) Fette Seifen Anstr. Mittel 80, 21-37.
17. Russell, F.S. (1976) in The Eggs and Planktonic Stages of British Marine Fishes, Academic Press, London, England.
18. Jangaard, P.M. (1974) Bull. Fish. Res. Board Canada No. 186.
19. Boulebache, H. (1981) Amer. Zool. 21, 377-389.
20. Vetter, R.D., Hodson, R.E., and Arnold, C. (1983) Can. J. Fish. Aquatic Sci. 40, 627-634.
21. Cetta, C.M. and Capuzzo, J.M. (1982) Mar. Biol. 71, 327-337.
22. Cell Calcium: Inositol Phospholipids and Cell Calcium (Michell R.H., ed.) Vol. 3, Churchill Livingstone, Edinburgh and New York.
23. Chacko, G.K., Goldman, D.E., and Pennock, B.E. (1972) Biochim. Biophys. Acta 280, 1-16.
24. Hazel, J.R. (1979) Amer. J. Physiol. 236, R91-R101.
25. Castledine, A.J., and Buckley, J.T. (1982) Comp. Biochem. Physiol. 71B, 119-126.
26. Cowey, C.B., and Sargent, J.R. (1979) in Fish Physiology, Vol. 8, (Hoar, W.S., Randall, D.J., and Brett, J.R., eds.) pp. 1-69, Academic Press, New York.

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Sterol Metabolism in the Nematode *Caenorhabditis elegans*

DAVID J. CHITWOOD,* WILLIAM R. LUSBY, RUBEN LOZANO, MALCOLM J. THOMPSON and **JAMES A. SVOBODA,** *Insect Physiology Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, MD 20705*

ABSTRACT

The metabolism of various dietary sterols and the effects of an azasteroid on sitosterol metabolism in the free-living nematode *Caenorhabditis elegans* was investigated. The major unesterified sterols of *C. elegans* in media supplemented with sitosterol, cholesterol or desmosterol included 7-dehydrocholesterol (66.5%, 40.5%, 31.2%, respectively), cholesterol (6.7%, 52.3%, 26.9%), lathosterol (4.4%, 3.6%, 1.7%) and 4α-methylcholest-8(14)-en-3β-ol (4.2%, 2.1%, 3.8%). Esterified sterols, representing less than 20% of the total sterols, were somewhat similar except for a significantly higher relative content of 4α-methylcholest-8(14)-en-3β-ol (23.3%, 23.4%, 10.6%). Thus *C. elegans* not only removes the substituent at C24 of dietary sitosterol but possesses the unusual ability to produce significant quantities of 4α-methylsterols. When *C. elegans* was propagated in medium supplemented with sitosterol plus 5 μg/ml of 25-azacoprostane hydrochloride, the azasteroid strongly interfered with reproduction and motility of *C. elegans* and strongly inhibited the Δ24-sterol reductase enzyme system; excluding sitosterol, the major free sterols of azacoprostane-treated *C. elegans* were cholesta-5,7,24-trien-3β-ol (47.9%), desmosterol (9.4%), fucosterol (2.1%) and cholesta-7,24-dien-3β-ol (2.0%). These 4 sterols are likely intermediates in the metabolism of sitosterol in *C. elegans.*
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INTRODUCTION

Although the sterol composition of several nematodes has been investigated, knowledge of nematode sterol metabolism has been limited by the complex nutritional requirements or parasitic habits of these organisms. Like insects, nematodes nutritionally require sterol because they lack the capacity for de novo sterol biosynthesis (1-6). Moreover, many azasteroids and nonsteroidal amines and amides that interfere with insect sterol metabolism and development also inhibit nematode growth and development (7-9) and thus provide model compounds for developing novel nematode control agents. The mode of action of these compounds in nematodes is unknown, however. In an initial investigation, we discovered that the free-living nematode, *Caenorhabditis elegans,* converts substantial quantities of dietary 4-desmethylsterols to 4α-methylsterol derivatives (10). The present study was undertaken to determine the pathways involved in the metabolism of various dietary sterols and to investigate the effects of an azasteroid on sterol metabolism in this nematode.

MATERIALS AND METHODS

Steroids

No impurities were detected in dietary cholesterol (cholest-5-en-3β-ol) or desmosterol (cholesta-5,24-dien-3β-ol) by either thin layer chromatography (TLC) or gas liquid chromatography (GLC). Although pure by TLC, GLC revealed sitosterol (24α-ethylcholest-5-en-3β-ol) containing 1.5% campesterol (24α-methylcholest-5-en-3β-ol). [26-^{14}C]Desmosterol was obtained from New England Nuclear, Boston, MA, and [4-^{14}C]sitosterol was purchased from Amersham Corp., Arlington Heights, IL. After column chromatographic purification, the radiochemical purity of each surpassed 99% by TLC and GLC. The appropriate amount of the proper nonradiolabeled sterol was added to create specific activities of 2200 dpm/μg, as determined by GLC. The 25-azacoprostane hydrochloride was previously synthesized (11). Authentic cholesta-5,7,9(11)-trien-3β-yl acetate was synthesized from 7-dehydrocholesteryl acetate (12).

Dietary sterols were initially dissolved in benzene in a centrifuge tube, Tween 80 was added at a rate of 20 mg sterol per ml of Tween 80, benzene was removed under a stream of nitrogen and sterol and Tween 80 were dispersed by addition of warm water with vigorous agitation. The 25-azacoprostane hydrochloride was similarly solubilized, except methanol was substituted for benzene. Concentrations of 25-azacoprostane hydrochloride in preliminary toxicity determinations varied between 1-100 μg/ml.

Nematode Culture

C. elegans was sterilely cultured at 22 C in an aqueous basal medium containing yeast extract, soy peptone, casein hydrolyzate and dextrose (13), with all ingredients except soy

*To whom correspondence should be addressed.

peptone extracted 3 times with chloroform/methanol (2:1, v/v) to remove any endogenous sterol. Filter-sterilized stock solutions (10X) of sterol (final concentration in media of 25 μg/ml, and chloroform-extracted hemoglobin (final concentration of 500 μg/ml) were added to autoclaved basal media. Living nematodes were isolated from logarithmic phase cultures (14).

Sterol Analysis

Lipids were isolated from lyophilized nematodes and separated into neutral and polar lipids (15). Crude free sterol and steryl ester fractions were isolated from the neutral lipids by silicic acid column chromatography (16). The 4-desmethylsterols and 4α-methylsterols were isolated from saponification products of each fraction, separated on silicic acid columns and identified and quantified by GLC. The sterols were analyzed further by acetylation, purification of the resulting steryl acetates on silicic acid columns, argentation column chromatography of the purified acetates (17) and GLC. Argentation TLC was performed on Silica Gel 60 high performance TLC plates (E. Merck, Darmstadt, W. Germany) previously impregnated with $AgNO_3$ (18). Plates were developed with a solvent system of hexane/benzene/chloroform/acetic acid (110:65:25:0.5, v/v/v/v). Identifications were made via gas chromatography-mass spectrometry (GC-MS) and ultraviolet (UV) and proton magnetic resonance (PMR) spectroscopy. Compounds were dissolved in methanol or $CDCl_3$ for UV or PMR spectroscopy, respectively.

Radioactivity determinations were performed with a toluene-based cocktail containing 0.5% PPO and 0.03% dimethyl POPOP. Quench was measured for each sample by an external standard, and counting efficiency was determined by reference to a standard curve generated from a 10-membered series of quenched standards (Packard Instrument Co., Downers Grove, IL).

Instrumentation

GLC was performed isothermally with a Varian model 3700 gas chromatograph equipped with packed glass columns (2 mm i.d. × 2 m) containing 2.0% SE-30 or 2.0% OV-17 liquid phases. UV spectra were recorded with a Perkin-Elmer model 559 double beam spectrophotometer, and PMR spectra were recorded at 60 mHz with a JEOL FX-60-Q Fourier Transform Instrument. GC-MS was performed in a Finnigan model 4510 instrument fitted with a 15 m × 0.32 mm DB-1 fused silica capillary column (0.25 μm film) and connected to an Incos data system. A Packard Tri-Carb 460 CD Liquid Scintillation System was employed for radioactivity determinations.

RESULTS AND DISCUSSION

The sterol content of *C. elegans* varied from 0.13% to 0.34% of nematode dry wt (Table 1). Although previously investigated nematodes have contained 0.01% (19) to 0.38% (20) sterol, comparison with other species is difficult because nematode sterol content has been shown to vary in direct proportion to dietary sterol concentration (2). Regardless of the dietary sterol, most of the sterol from *C. elegans* was nonesterified as esterified sterol accounted for only 13% to 19% of the total sterol (Table 1).

The sterols from free and esterified sterol fractions of *C. elegans* propagated in media containing sitosterol, cholesterol or desmosterol are listed in Table 2. Excluding dietary sterol, 7-dehydrocholesterol (cholesta-5,7-dien-3β-ol) was by far the major sterol, accompanied by lesser quantities of cholesterol, lathosterol (cholest-7-en-3β-ol) and 4α-methylcholest-8(14)-en-3β-ol. All 4 sterols were identical to authentic standards by GLC (Table 3), GC-MS and, for 7-dehydrocholesterol, UV spectroscopy (λ_{max} at 272, 282 and 294 nm). The abundance of

TABLE 1

Sterol Content of *Caenorhabditis elegans* Propagated in Media Containing 25 μg/ml Different Dietary Sterols

	Supplemented sterol			
	Cholesterol	Desmosterol	Sitosterol	Sitosterol plus 5 μg/ml 25-azacoprostane hydrochloride
Percentage of dry wt as sterol	0.17	0.34	0.13	0.19
Percentage of total sterol as esterified sterol	18.8	15.3	13.3	15.0

TABLE 2

Relative Percentages of Sterols in Free Sterol (FS) and Steryl Ester (SE) Fractions from *Caenorhabditis elegans* Propagated with Different Dietary Sterols[a]

	Supplemented sterol							
	Cholesterol		Desmosterol		Sitosterol		Sitosterol plus 5 μg/ml 25-azacoprostane hydrochloride	
	FS	SE	FS	SE	FS	SE	FS	SE
Cholesterol	52.3	41.2	26.9	18.7	6.7	9.3	0.4	0.3
7-Dehydrocholesterol	40.5	26.7	31.2	39.0	66.5	30.5	1.2	0.9
Lathosterol	3.6	5.7	1.7	1.4	4.4	3.6	0.2	0.3
Cholesta-5,7,9(11)-trien-3β-ol	1.4	1.5	1.4	1.4	0.8	0.3	0.1	0.1
Sitosterol	–	–	–	–	16.0	30.3	27.5	42.7
Campesterol	–	–	–	–	0.7	1.3	0.8	1.4
Desmosterol	–	–	32.3	22.8	–	–	9.4	6.8
Cholesta-5,7,24-trien-3β-ol	–	–	2.4	4.5	–	–	47.9	24.9
Fucosterol	–	–	–	–	0.1	0.1	2.1	3.6
Cholesta-5,7,9(11),24-tetraen-3β-ol	–	–	–	–	–	–	1.1	1.9
Cholesta-7,24-dien-3β-ol	–	–	–	–	–	–	2.0	2.6
Cholest-24-en-3β-ol	–	–	–	–	–	–	0.1	0.3
24-Methylenecholesterol	–	–	–	–	–	–	0.1	–
Campesta-7,24(28)-dien-3β-ol	–	–	–	–	–	–	0.1	0.2
Campesta-5,7-dien-3β-ol	–	–	–	–	–	–	0.1	0.1
Stigmasta-5,7-dien-3β-ol	–	–	–	–	–	–	0.1	0.1
Other 4-desmethylsterols	–	–	–	–	–	–	1.3	0.1
4α-Methylcholest-8(14)-en-3β-ol	2.1	23.4	3.8	10.6	4.2	23.3	0.2	0.2
4α-Methylcholest-7-en-3β-ol	0.1	1.3	0.3	1.6	0.7	1.4	0.5	0.3
4α-Methylcholesta-8(14),24-dien-3β-ol	–	–	–	–	–	–	1.8	6.3
4α-Methylcholesta-7,24-dien-3β-ol	–	–	–	–	–	–	2.4	6.8
4α-Methylcholestan-3β-ol	–	–	–	–	–	–	0.1	0.1
4α-Methylstigmastan-3β-ol	–	–	–	–	–	–	0.2	0.1

[a]The 4α-methylsterol data is from a previous report (Chitwood et al., Steroids, 42, 311-319.

7-dehydrocholesterol, which was observed especially in sitosterol-fed nematodes, indicates that sterol metabolism in *C. elegans* may be centered around the production of 7-dehydrocholesterol rather than cholesterol and that this production may be favored when the dietary sterol is a phytosterol. Cholesterol, 7-dehydrocholesterol and lathosterol are the major sterols of another free-living nematode, *Turbatrix aceti* (2). Identification of lathosterol in *C. elegans*, together with our previous findings that *C. elegans* produces Δ7- and Δ8(14)-4α-methylsterols from 4-desmethylsterol precursors (10), indicates that lathosterol may be an important precursor in 4α-methylsterol biosynthesis in *C. elegans*.

In addition, small quantities of cholesta-5,7,9(11)-trien-3β-ol were identified by a UV spectrum of the acetate (λ_{max} at 311, 325 and 340 nm) that was identical to a literature spectrum (21) as well as that of our synthesized reference compound. During argentation TLC, the synthesized acetate and the acetate derived from *C. elegans* behaved identically, migrating between cholesteryl and 7-dehydrocholesteryl acetates, as has been previously described (12). The authentic compound and the acetate derived from *C. elegans* were also identical by GLC (Table 3) and by GC-MS: m/z (rel.intensity): 424 (molecular ion M^+, 5%), 364 (M-CH_3COOH, 100), 349 (M-CH_3COOH-CH_3, 33), 251 (M-CH_3COOH-C_8H_{17}, 31), 209 (M-CH_3-$COOH$-$C_{11}H_{23}$, 64), 197 (M-CH_3COOH-C_{12}-H_{23}, 43), and 195 (M-CH_3COOH-$C_{12}H_{25}$, 52). Possibly an artifact, cholesta-5,7,9(11)-trien-3β-ol has not previously been detected in nematodes. A sterol with the unusual Δ5,7,9(11) nucleus (ergosta-5,7,9(11),22-tetraen-3β-ol) has been detected in the fungus *Chaetomium cochliodes* (22).

In addition, desmosterol-fed *C. elegans* contained small amounts of a sterol with UV λ_{max} at 272, 282 and 294 nm, indicative of a Δ5,7-sterol. The steryl acetate migrated more slowly during argentation TLC than 7-dehydrocholesteryl acetate. The MS of the acetate [424 (M^+, 3%), 364 (M-CH_3COOH, 98), 349 (M-CH_3-$COOH$-CH_3, 20), 253 (M-CH_3COOH-C_8H_{15}, 16), 251 (M-CH_3COOH-C_8H_{15}-2H, 10), and 69 (C_5H_9, 100)] was similar to that reported for cholesta-5,7,24-trien-3β-yl acetate in the literature (23). In addition, GLC relative retention times (RRT, Table 3) were identical to values

TABLE 3

Gas Liquid Chromatographic Relative Retention Times of Sterols from *Caenorhabditis elegans*, Expressed Relative to Cholesterol

	SE-30	OV-17
Cholesta-5,7,9(11)-trien-3β-ol	0.98	1.06
Cholesterol	1.00	1.00
Cholesta-5,7,9(11),24-tetraen-3β-ol	1.07	1.28
Desmosterol	1.09	1.21
7-Dehydrocholesterol	1.10	1.16
Cholest-24-en-3β-ol	1.12	1.26
Lathosterol	1.12	1.19
Cholesta-5,7,24-trien-3β-ol	1.21	1.42
Cholesta-7,24-dien-3β-ol	1.23	1.44
24-Methylenecholesterol	1.26	1.36
Campesterol	1.30	1.33
Campesta-7,24(28)-dien-3β-ol	1.42	1.63
Campesta-5,7-dien-3β-ol	1.43	1.52
Sitosterol	1.63	1.67
Fucosterol	1.63	1.75
Stigmasta-5,7-dien-3β-ol	1.78	1.87
4α-Methylcholest-8(14)-en-3β-ol	1.18	1.16
4α-Methylcholest-7-en-3β-ol	1.31	1.37
4α-Methylcholesta-8(14),24-dien-3β-ol	1.29	1.41
4α-Methylcholesta-7,24-dien-3β-ol	1.44	1.66
4α-Methylcholestan-3β-ol	1.20	1.18
4α-Methylstigmastan-3β-ol	1.95	1.90

GLC was performed isothermally on packed glass columns (2 m × 2 mm i.d.) containing 2.0% SE-30 or 2.0% OV-17 stationary phases. The 4α-methylsterol data is from a previous report (Chitwood et al., Steroids, 42, 311-319).

calculated from lathosterol and desmosterol standards. Cholesta-5,7,24-trien-3β-ol is another sterol that has not previously been detected in nematodes.

When radiolabeled desmosterol or sitosterol was the dietary sterol, all sterols in both the free sterol and steryl ester fractions from *C. elegans* contained approximately the same specific activity as the original dietary sterol. This indicates that all sterols isolated from *C. elegans* were derived from the supplemented dietary sterol and were not concentrated media contaminants. *C. elegans*, therefore, dealkylated the plant sterol, sitosterol, and converted it to 3 other sterols probably better suited for its own growth and reproduction: 7-dehydrocholesterol, cholesterol and lathosterol. The results also provide additional evidence for the lack of de novo sterol biosynthesis in *Caenorhabditis* (4).

A few conspicuous differences occurred between the free and the esterified sterol fractions. Foremost among these was the detection of surprisingly high amounts of 4α-methylsterols in the steryl esters of *C. elegans*. Highly speculative explanations for this phenomenon would include an esterification requirement for 4α-methylsterol synthesis or transport or possibly a specific hormonal, pheromonal or other physiologic role for a 4α-methylsteryl ester or metabolite. In addition, a larger proportion of sitosterol occurred in the steryl esters of *C. elegans*. A similar sequestration of phytosterols in steryl esters has been reported in the plant-parasitic nematode *Ditylenchus dipsaci* (24).

Under our described cultural conditions, *C. elegans* normally moves rapidly in a sinusoidal manner. When ca. 1000 nematodes were transferred into 1 ml of media supplemented with 25-azacoprostane hydrochloride, no immediate abnormalities were apparent, even at a 100 μg/ml concentration of inhibitor. After 3 days, however, cultures with a 20 μg/ml concentration or greater of azasteroid contained fewer moving nematodes, and their movements were slow and erratic. After 4 days, reproduction was obviously poorer in azasteroid-treated cultures. Effects on both reproduction and motility were directly related to inhibitor concentration, with half-maximal reproductive rate occurring between 4-6 μg/ml of inhibitor. Reproduction was reduced somewhat at concentrations as low as 2 μg/ml, and even though the reproductive rate was slow, reproduction continued at concentrations as high as 100 μg/ml.

Treatment of *C. elegans* with 5 μg/ml of 25-azacoprostane hydrochloride resulted in slight hyperlipidemia (Table 4). Compared

TABLE 4

Lipid Content of *Caenorhabditis elegans* Propagated with Different Dietary Sterols, Expressed as Relative Percentage of Nematode Dry Weight

	Supplemented sterol			
Lipid class	Cholesterol	Desmosterol	Sitosterol	Sitosterol plus 5 μg/ml 25-azacoprostane hydrochloride
Total lipid	17.8	17.2	18.3	22.2
Neutral lipid	9.4	8.9	9.4	11.8
Polar lipid	8.4	8.3	8.9	10.4

with untreated nematodes, azacoprostane-treated nematodes contained very small quantities of cholesterol, 7-dehydrocholesterol and lathosterol (Table 2). The major sterol had GLC, GC-MS and UV absorbance properties identical to the cholesta-5,7,24-trien-3β-ol previously isolated from nematodes fed desmosterol. The PMR spectrum of the acetate contained the same C18 and C19 methyl resonances (δ 0.63 ppm and δ 0.98 ppm, respectively) as a 7-dehydrocholesteryl acetate standard and also contained the C26, C27 doublet at δ 2.68 ppm present in the spectrum of authentic desmosteryl acetate. The major sterol in azacoprostane-treated nematodes was thus identified as cholesta-5,7,24-trien-3β-ol; its abundance indicates that the azasteroid significantly inhibited Δ24-sterol reductase in *C. elegans.*

Azasteroid-treated *C. elegans* contained several other sterols that occurred in no more than trace quantities in sitosterol-propagated controls. Fucosterol (stigmasta-5,24(28)E-dien-3β-ol), 24-methylenecholesterol (campesta-5,24-(28)-dien-3β-ol), desmosterol, campesta-5,7-dien-3β-ol and stigmasta-5,7-dien-3β-ol were identified following argentation column chromatography of their acetates and were identical to authentic reference compounds by argentation TLC, GLC (Table 3) and GC-MS.

Although lacking reference compounds, we did identify in azasteroid-treated nematodes the following compounds as steryl acetate derivatives by GC-MS and by GLC RRT (Table 3) deviating no more than 0.01 unit from calculated values: cholesta-5,7,9(11),24-tetraen-3β-ol, cholesta-7,24-dien-3β-ol, cholest-24-en-3β-ol and campesta-7,24(28)-dien-3β-ol. None of these have previously been detected in nematodes. The first of these sterols as the acetate had a MS that included an M^+ of m/z 422 (4%) and fragments at 362 (M-CH_3COOH, 68), 347 (M-CH_3COOH-CH_3, 15), 251 (M-CH_3COOH-C_8H_{15}, 18), 249 (M-CH_3COOH-C_8H_{15}-2H, 14), 209 (M-CH_3COOH-$C_{11}H_{21}$, 44), 197 (M-CH_3-$COOH$-$C_{12}H_{21}$, 29), 195 (M-CH_3COOH-$C_{12}H_{23}$, 34) and 69 (C_5H_9, 100). Cholesta-7,24-dien-3β-yl acetate had a PMR spectrum with C18 and C19 methyl resonances at δ 0.54 and δ 0.82 ppm, respectively, as did the PMR spectrum of a lathosteryl acetate reference. The MS of this diene was similar to that of a literature spectrum (25) and included an M^+ of m/z 426 (12%) and fragments at 411 (M-CH_3, 10), 366 (M-CH_3COOH, 2), 351 (M-CH_3COOH-CH_3, 3), 342 (M-C_5H_9-CH_3, 13), 315 (M-C_8H_{15}, 3), 313 (M-C_8H_{15}-2H, 91), 255 (M-CH_3COOH-C_8H_{15}, 10), 253 (M-CH_3COOH-C_8H_{15}-2H, 4), 229 (M-CH_3COOH-$C_{10}H_{17}$, 6), 213 (M-CH_3COOH-$C_{11}H_{21}$, 19) and 69 (C_5H_9, 100). Cholest-24-en-3β-yl acetate possessed a MS including an M^+ of 428 (4%) and fragments at 413 (8), 368 (0.4), 353 (4), 344 (12), 317 (3), 315 (70), 257 (6), 255 (43), 215 (17) and 69 (100). The MS of campesta-7,24(28)-dien-3β-yl acetate derived from *C. elegans* [m/z 440 (M^+, 3%), 425 (M-CH_3, 3), 365 (M-CH_3COOH-CH_3, 2), 356 (M-C_6H_{12}, 22), 315 (M-C_9H_{17}, 2), 313 (M-C_9H_{17}-2H, 100), 255 (M-CH_3COOH-C_9H_{17}, 14), 253 (M-CH_3COOH-C_9H_{17}-2H, 9), and 213 (M-CH_3COOH-$C_{12}H_{23}$, 26)] was identical to that of the same compound we isolated from corn pollen (26).

Excluding dietary sterol, 96.6% of the total sterol in azacoprostane-treated nematodes contained a C24 double bond. Accumulation of fucosterol, desmosterol, cholesta-5,7,24-trien-3β-ol and cholesta-7,24-dien-3β-ol indicates that these 4 sterols are metabolic intermediates in the metabolism of sitosterol by *C. elegans* (Fig. 1). Similarly, detection of 24-methylenecholesterol and campesta-7,24(28)-dien-3β-ol indicates that these 2 sterols are probable intermediates in campesterol metabolism in *C. elegans,* as small amounts of this sterol did contaminate the dietary sitosterol. We have previously reported (10) on the inhibition of a Δ7-sterol isomerase in *C. elegans* by 25-azaco-

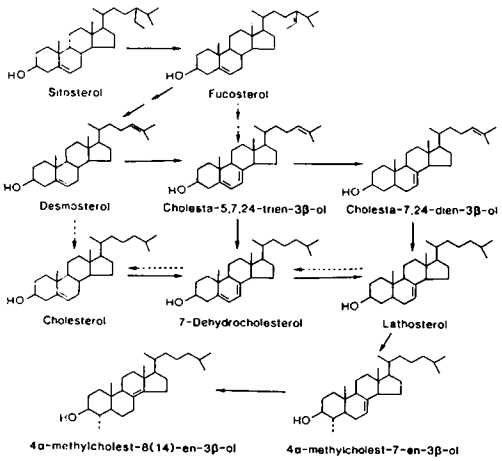

FIG. 1. Metabolism of sitosterol by *Caenorhabditis elegans*. Dotted lines indicate possible but unproven metabolic steps.

prostane hydrochloride and the resultant accumulation of 4α-methylcholesta-7,24-dien-3β-ol (Table 2). All sterols isolated from azacoprostane-treated nematodes were radiolabeled and had specific activities quite similar to the original dietary sitosterol, with the exception of compounds for which quantities were insufficient for determination: cholest-24-en-3β-ol, campesta-5,7-dien-3β-ol, stigmasta-5,7-dien-3β-ol, 4α-methylcholestan-3β-ol and 4α-methylstigmastan-3β-ol.

Most plant-feeding insects produce cholesterol by removing the C24 methyl or ethyl groups typical of plant sterols (27). Treatment of such insects with azasteroids interferes with cholesterol production by inhibition of Δ24-sterol reductase and results in the accumulation of Δ24-sterols (11,28). Because of the abundance of Δ24-sterols in azacoprostane-treated *C. elegans*, the phytosterol dealkylation mechanism in *C. elegans* must be very similar to that in insects. Indeed, predominance of 7-dehydrocholesterol and accumulation of cholesta-5,7,24-trien-3β-ol, fucosterol and desmosterol when fed sitosterol plus azasteroid are features shared by both *C. elegans* and the confused flour beetle, *Tribolium confusum* (29,30).

With the exception of the free-living *T. aceti*, phytosterol dealkylation in other nematodes has received limited attention, largely because of the difficulty in culturing parasitic nematodes apart from their plant or animal hosts. The animal parasite, *Ascaris lumbricoides*, did not dealkylate or otherwise metabolize injected radiolabeled sitosterol (31). Because the few plant-parasitic nematodes investigated thus far have contained much greater amounts of cholesterol and/or lathosterol than their hosts (24, 32), phytoparasitic nematodes have been suggested as being capable of C24 dealkylation (32). The toxicity of azasteroids to nematodes, together with our findings that 25-azacoprostane hydrochloride interferes with phytosterol dealkylation by inhibiting Δ24-sterol reductase in *C. elegans*, indicates that steroid metabolism is a viable target for novel nematicidal agents.

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REFERENCES

1. Dutky, S.R., Robbins, W.E., and Thompson, J.V. (1967) Nematologica 13, 140.
2. Cole, R.J., and Krusberg, L.R. (1968) Life Sci. 7, 713-724.
3. Hieb, W.F., and Rothstein, M. (1968) Science 160, 778-780.
4. Rothstein, M. (1968) Comp. Biochem. Physiol. 27, 309-317.
5. Bolla, R. (1979) J. Nematol. 11, 250-259.
6. Comley, J.C.W., and Jaffe, J.J. (1981) J. Parasitol. 67, 609-616.
7. Feldmesser, J., Thompson, M.J., Robbins, W.E., and Sponaugle, R.P. (1976) Experientia 32, 466-467.
8. Douvres, F.W., Thompson, M.J., and Robbins, W.E. (1980) Vet. Parasitol. 7, 195-205.
9. Bottjer, K.P., Weinstein, P.P., and Thompson, M.J. (1980) J. Parasitol. Suppl. 66, 52 (abstr.).
10. Chitwood, D.J., Lusby, W.R., Lozano, R., Thompson, M.J., and Svoboda, J.A. (1983) Steroids, 42, 311-319.
11. Svoboda, J.A., Thompson, M.J., and Robbins, W.E. (1972) Lipids 7, 553-556.
12. Wilton, D.C. (1982) Biochem. J. 208, 521-523.
13. Vanfleteren, J.R. (1976) Experientia 32, 1087-1088.
14. Fletcher, C.L., and Krusberg, L.R. (1973) Comp. Biochem. Physiol. 45B, 159-165.
15. Chitwood, D.J., and Krusberg, L.R. (1981) Comp. Biochem. Physiol. 69B, 115-120.
16. Thompson, M.J., Patterson, G.W., Dutky, S.R., Svoboda, J.A., and Kaplanis, J.N. (1980) Lipids 15, 719-733.
17. Vroman, H.E., and Cohen, C.F. (1967) J. Lipid Res. 8, 150-152.
18. Sliwowski, J.K., and Caspi, E. (1977) J. Steroid Biochem. 8, 47-49.
19. Orcutt, D.M., Fox, J.A., and Jake, C.A. (1978) J. Nematol. 10, 264-269.
20. Cole, R.J., and Krusberg, L.R. (1967) Comp. Biochem. Physiol. 21, 109-114.
21. Antonucci, R., Bernstein, S., Giancola, D., and Sax, K.J. (1951) J. Org. Chem. 16, 1159-1164.
22. Safe, S., and Brewer, D. (1973) Lipids 8, 311-314.
23. Galli, G., and Maroni, S. (1967) Steroids 10, 189-197.
24. Cole, R.J., and Krusberg, L.R. (1967) Exp. Parasitol. 21, 232-239.

25. Parks, L.W., Anding, C., and Ourisson, G. (1974) Eur. J. Biochem. 43, 451-458.
26. Svoboda, J.A., Herbert, E.W., Lusby, W.R., and Thompson, M.J. (1983) Arch. Insect Biochem. Physiol. 1, 25-31.
27. Svoboda, J.A., Kaplanis, J.N., Robbins, W.E., and Thompson, M.J. (1975) Ann. Rev. Entomol. 20, 205-220.
28. Svoboda, J.A., and W.E. Robbins (1971) Lipids 6, 113-119.
29. Svoboda, J.A., Robbins, W.E., Cohen, C.F., and Shortino, T.J. (1972) in Insect and Mite Nutrition (Rodriguez, J.G., ed.) pp. 505-516, North Holland, Amsterdam, The Netherlands.
30. Svoboda, J.A., Thompson, M.J., Robbins, W.E., and Kaplanis, J.N. (1978) Lipids 13, 742-753.
31. Barrett, J., Cain, G.D., and Fairbairn, D. (1970) J. Parasitol. 56, 1004-1008.
32. Svoboda, J.A., and Rebois, R.V. (1977) J. Nematol. 9, 286 (abstr.).

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Preferential Metabolism of Linoleic Acid by Five-Day-Old Barley Shoots

LOLA A. NABORS,[a] MARJORIE S. MORGAN,[b] DAVID W. NEWMAN[a] and JAN G. JAWORSKI,[b]* [a]*Botany Department, and* [b]*Chemistry Department, Miami University, Oxford, OH 45056*

ABSTRACT

The degradation of exogenous radioactively labeled fatty acids by 5-day-old barley shoots was examined. [1-^{14}C] Linoleic acid was observed to be degraded 7 times faster than [1-^{14}C] oleic acid and 5 times faster than [1-^{14}C] palmitic acid. The pathway of degradation was determined by identifying the water-soluble products and determined to be β-oxidation. During a 15 min incubation, the barley shoots took up 0.91 nmol/g fresh wt of linoleic acid, of which 0.16 nmol/g fresh wt was incorporated into glutamic acid, 0.07 nmol/g fresh wt into succinic acid and 0.002 nmol/g fresh wt into carbohydrates. By 30 min, additional TCA cycle intermediates, especially malic acid, were detected. Palmitic acid and oleic acid were broken down to the same products. The rates of uptake and the distribution of label into lipids were determined. The uptake of label by the tissue was similar for all 3 fatty acid substrates. Label from linoleic, oleic and palmitic acids was found to be incorporated into similar lipids, primarily phosphatidylcholine (PC), and the extent of incorporation was comparable. Although all 3 fatty acid substrates were broken down by β-oxidation, the reason for the more rapid degradation of linoleic acid was not established. It does not appear to be related to uptake of substrate or incorporation of label into lipids.
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INTRODUCTION

Fatty acids play an important role as energy-rich fuel in mammalian systems. The fatty acids are stored in the form of triacylglycerols in specialized tissues devoted to fat storage and metabolism. In general, the green tissue of plants does not store fat. Triacylglycerols are found in relatively small amounts in vegetative tissues; therefore fatty acids are not believed to be a major source of energy in the photosynthetic organs of plants. The seeds of certain plants, however, do store a considerable amount of triacylglycerols and much of what is known about fatty acid metabolism in plants comes from studies conducted on these oil-rich seeds. At this time, further work with other tissues is necessary to adequately assess the catabolism of fatty acids in plants.

Three major degradative processes for fatty acids have been established in various plant tissues: α-oxidation, β-oxidation, and peroxidation by lipoxygenase. The α-oxidation system is responsible for the oxidation of free C_n fatty acids to C_{n-1} aldehydes with liberation of CO_2. The aldehyde may be oxidized to the corresponding C_{n-1} fatty acid, which can then reenter the α-oxidation cycle. The physiological role of α-oxidation in plants is not fully defined. Hitchcock and James (3) proposed that α-oxidation may be a significant metabolic pathway in growing plants. They found in young leafy tissue, fatty acids were more readily oxidized by α-oxidation than β-oxidation, and on leaf maturation this activity was lost. In addition, research has suggested that α-oxidation is the main source of odd-numbered fatty acids, because they can be formed from the more common even-numbered fatty acids (4). Although not established, α-oxidation may also be responsible for the formation of 2-D-hydroxy acids found in specialized complex lipids, e.g., cerebrosides.

Another important degradative mechanism is peroxidation by lipoxygenase. The exact process is unclear, but this group of enzymes is known to catalyze the oxygenation, by molecular oxygen, of fatty acids containing a *cis,cis*-1,4-pentadiene system to form conjugated *cis,trans*-hydroperoxydiene derivatives. A characteristic substrate is 18:2 as lipoxygenase prefers free polyunsaturated fatty acids. A variety of products exist, including hydroperoxides and the so-called leaf alcohols and aldehydes, that are volatile and give characteristic odors to various plants (2).

Stumpf and Barber (6) provided the first evidence for in vitro β-oxidation in plants while studying germinating oil-rich seeds. In general, the mechanism was found to be similar to β-oxidation in mammalian systems. Unlike the mammalian system, β-oxidation in plants was found to be extramitochondrial (7,8). In 1969, Cooper and Beevers demonstrated that the major β-oxidation process of germinating fatty

*To whom correspondence should be addressed.

seeds resides in the glyoxysomes (9,10). In 1981, Gerhardt showed that the β-oxidation enzymes were associated with peroxisomes in spinach leaves (11). Macey and Stumpf (12), while working on artichoke tuber tissue, also found the β-oxidation enzymes present in microbodies and not in the mitochondria.

Cooper and Beevers (10) showed the acetyl-CoA produced by glyoxysomal β-oxidation was consumed in the glyoxylate cycle, which is also found in this organelle (9,13). They suggested that β-oxidation in germinating oil-rich seeds was involved in the efficient conversion of oils to sucrose by channeling acetate produced by β-oxidation through the glyoxylate cycle. That this is the major role of β-oxidation in these tissues is now generally accepted (2).

The physiological role of β-oxidation in other plant tissues is not as well defined. Macey and Stumpf (12), in a recent study on Jerusalem artichokes, reported they were unable to detect the presence of the glyoxylate cycle enzymes. Thus, acetyl-CoA must have another fate in this tissue. They suggested the product of β-oxidation in nongluconeogenic tissues may be a substrate for fatty acid synthesis in plastids or for further oxidation in the mitochondria.

In a series of experiments carried out in this laboratory in which free fatty acids were incubated with 5-day old barley shoots, we noted a consistently rapid loss of 18:2. We present evidence here that in these barley shoots, there was a preferential degradation of 18:2 and that the pathway of degradation was β-oxidation. The resultant acetyl-CoA was then incorporated into organic and amino acids.

MATERIALS AND METHODS

Plant Growth Conditions

Barley (*Hordeum vulgare* L. cv. Atlas 68) seeds were planted in vermiculite and grown for 5 days in a growth chamber with cycles of 16 hr light (3.2 mW cm^{-2} from fluorescent lamps) at 22 C and 8 hr dark at 20 C. The plants were irrigated daily with distilled water.

Materials

$^{14}CO_2$ (53 mCi/mmol), [^{14}C] acetic acid (59 mCi/mmol), [1-^{14}C] linoleic acid (54.7 mCi/mmol) and [1-^{14}C] oleic acid (52.3 mCi/mmol) were purchased from New England Nuclear, Boston, MA. [1-^{14}C] Palmitic acid (56 mCi/mmol) was obtained from Amersham Corporation, Arlington Heights, IL. BSTFA was obtained from Pierce Chemical Company, Rockford, IL. The barley seeds (Atlas 68) were generously provided by Dr. Jerry McClure, Botany Department, Miami University, Oxford, OH.

Tissue Incubation

Five-day-old barley shoots were excised above the coleoptile and the lower surface gently abraded with carborundum to remove the epidermis. The leaves were rinsed in distilled water to remove the carborundum, gently blotted dry and weighed (20 shoots; ca. 0.9 g fresh wt). The shoots were then floated in Petri dishes containing 20 ml distilled water and 100 μl substrate. The substrates, [1-^{14}C] acetic acid, [1-^{14}C] palmitic acid, [1-^{14}C] oleic acid and [1-^{14}C] linoleic acid, were prepared by dissolving 1 μCi (17.8 nmol) of each in 20 μl 1,2-propanediol and diluting with 80 μl distilled water. With the lower surface of the leaf contacting the medium, the tissue was incubated for the specified period. When $^{14}CO_2$ was used, the tissue was placed on wet filter paper in a petri dish. The dish was placed in a 3 l dessicator and 100 μCi $^{14}CO_2$ was released by acidification with sulfuric acid of a [^{14}C] sodium bicarbonate solution. Normally, incubations were carried out at 22 C in the same light as indicated for growth conditions. For dark experiments, immediately after adding substrate the petri dishes were wrapped in aluminum foil and placed in a dark chamber at 22 C. At the end of each incubation time, the shoots were removed from the labeled medium, rinsed with distilled water and placed in a test tube containing warm chloroform/methanol (1:1, v/v). The tube was sealed and the tissue was extracted by heating at 60 C for 30 min. The solution was cooled and transferred to another test tube and the volume reduced to 1 ml under a stream of nitrogen while heating at 60 C. Distilled water was added to produce (a) an organic phase (chloroform) containing lipids and (b) an aqueous phase (water/methanol) containing water-soluble breakdown products. The mixture was centrifuged and the phases separated.

Analytical Methods

The amounts of radioactivity in the aqueous and chloroform phases were measured by liquid scintillation spectrometry. The scintillation cocktail was: toluene/Triton X-100 (3:1, v/v); 0.3% (w/v) 2,5-diphenyloxazole (PPO), and 0.02% 1,4-bis [2,(5-phenyloxazolyl)] benzene (POPOP).

The aqueous phase was analyzed by modifying procedures described by Stumpf and Burris (14). Columns were prepared with a bed height of 6 cm and i.d. of 0.6 cm. The aqueous solu-

tion was eluted sequentially through a Dowex 1 column and a Dowex 50 column. An additional 2 ml of distilled water was passed through each column to remove residual carbohydrates, which were not adsorbed to either column. The organic acids were eluted from Dowex 1 with 3 ml 16N formic acid and the amino acids were eluted off Dowex 50 with 3 ml 6N ammonium hydroxide. The amount of radioactivity in the eluents was measured as described above.

The eluents were evaporated to dryness and a known amount of phenanthrene (internal standard) was added to each sample. The organic acid samples were trimethysilylated with 100 μl BSTFA and placed in a heating block at 60 C for 30 min before injection into the gas chromatograph (GC). The amino acid samples were trimethylsilated by adding 100 μl BSTFA and heating the solution for 2.5 hr at 135 C in an oil bath (15).

The derivatized compounds were analyzed on a GC (Packard Model 427) equipped with a flame ionization detector (FID). The column used was a 2 m × 6 mm glass coil packed with 3% SE-30. The temperature program was 90-240 C with a 3 C rise/min. The GC was also equipped with a stream splitter and a radioactivity monitor (Packard Model 894, Packard Instrument Company, Inc., Downers Grove, IL) connected to an electronic integrator.

The chloroform phase samples were dried under N_2, redissolved in 1 ml chloroform and the lipids separated on 1 ml silicic acid columns. Neutral lipids and free fatty acids were eluted with 10 ml chloroform. This was followed by 10 ml acetone to remove glycolipids and then 10 ml methanol to elute phospholipids.

The individual lipids were separated by thin layer chromatography (TLC) on Silica gel G. The solvent used to resolve neutral lipids and free fatty acids was petroleum ether/diethyl ether/acetic acid (80:20:1, v/v/v). The glycolipids were separated with chloroform/methanol/acetic acid/water (170:15:15:2, v/v/v/v) and the phospholipids with chloroform/methanol/acetic acid/water (170:30:20:7, v/v/v/v). Radioactivity was located with a Berthold TLC radioactivity scanner. Lipids were visualized with iodine vapor.

Electron Microscopy

Five-day old barley shoots were excised above the coleoptile and the lower epidermis gently abraded with carborundum. The leaves were rinsed with distilled water and allowed to remain in distilled water for 2 hr. The leaves were prepared for electron microscopy by a modification of procedures described by Mohr and Cocking (16). The leaves were fixed for 3 hr at 4 C with 2% glutaraldehyde in 0.05 M piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES) buffer (pH 7.0) and postfixed for 3 hr at 4 C with 2% OsO_4 dissolved in the same medium. A 7% uranyl acetate solution was added to the leaves for 30 min to enhance contrast. The samples were dehydrated at 4 C in acetone. The tissue was embedded in Spur's resin and sectioned with a diamond knife. The silver sections were stained with 7% uranyl acetate in 50% ethanol for 15 min and counterstained with 3% lead citrate for 10 min.

RESULTS

Incubation Conditions

Experiments were conducted to compare the uptake and metabolism of radioactively labeled 18:2, 18:1 and 16:0 by 5-day-old barley shoots, with emphasis on the degradation of these fatty acids. The carborundum treatment enhanced substrate incorporation but did not significantly change the labeling pattern of the products (17). In addition, electron micrographs of 5-day-old barley shoots abraded with carborundum and left in distilled water for 2 hr before incubation showed no apparent morphologic changes in the mesophyll cells.

The barley leaf tissue was incubated with the individual substrates for 0.25, 0.5, 1 and 2 hr (Table 1). The initial rate of uptake was more rapid for 18:1 than for the other 2 substrates. By 2 hr, however, similar molar quantities of 18:1, 18:2 and 16:0 were taken up by the tissue.

Degradation of Linoleic, Oleic and Palmitic Acids

As early as 0.25 hr, the radioactive label taken up by the barley shoots was found to be associated with water-soluble compounds using all 3 substrates (Table 2). The amount of label in the aqueous phase, however, was low for tissues incubated using either 18:1 or 16:0 substrates compared with 18:2. At 0.25 hr, 0.23 nmol/g fresh wt of 18:2 were metabolized into water-soluble compounds, whereas 0.06 nmol/g fresh wt and 0.04 nmol/g fresh wt of 18:1 and 16:0, respectively, were metabolized. The incorporation of label increases with time for all 3 substrates. By 2 hr, 0.24 nmol/g fresh wt of 18:1 and 0.36 nmol/g fresh wt of 16:0 were metabolized, where 1.74 nmol/g fresh wt of 18:2 were metabolized. Thus, although no preference was found for which substrate enters the tissue, once in the tissue, 18:2 was preferentially degraded to water-soluble products.

TABLE 1

The Uptake of Substrate by Five-Day-Old Barley Shoots

Substrate	Incubation time (hr)	Total uptake[a] (nmol/g fresh wt)	Uptake (% ^{14}C)
[1-^{14}C] 18:2	0.25	0.91 ± 0.45[b]	5.1
	0.50	1.59 ± 0.78	8.9
	1.0	2.33 ± 0.75	13.1
	2.0	3.86 ± 1.22	21.7
[1-^{14}C] 18:1	0.25	2.72 ± 0.43	15.3
	0.50	2.00 ± 0.78	11.2
	1.0	3.40 ± 1.13	19.1
	2.0	4.23 ± 1.52	23.8
[1-^{14}C] 16:0	0.25	0.97 ± 0.45	5.4
	0.50	2.06 ± 0.50	11.6
	1.0	3.33 ± 1.23	18.7
	2.0	4.11 ± 1.14	23.1

All incubations contained 20 shoots (ca. 0.9 g fresh wt). The shoots were incubated for the times indicated with 17.8 nmoles of the indicated substrate.
[a]Sum of the aqueous and organic fractions.
[b]Mean ± standard deviation; minimum of four experiments.

TABLE 2

The Distribution of Label into the Aqueous and Organic Phases

Substrate	Incubation time (hr)	Uptake		Uptake	
		Aqueous (nmol/g fresh wt)	Organic (nmol/g fresh wt)	Aqueous (% ^{14}C)	Organic (% ^{14}C)
[1-^{14}C] 18:2	0.25	0.23 ± 0.05[a]	0.68 ± 0.41	25.3	74.7
	0.50	0.49 ± 0.13	1.10 ± 0.78	30.8	69.2
	1.0	0.90 ± 0.35	1.53 ± 0.62	34.3	65.7
	2.0	1.74 ± 0.89	2.12 ± 1.12	45.1	54.9
[1-^{14}C] 18:1	0.25	0.06 ± 0.03	2.66 ± 0.46	2.2	97.8
	0.50	0.09 ± 0.06	1.94 ± 0.73	4.5	95.5
	1.0	0.18 ± 0.06	3.22 ± 1.07	5.3	94.7
	2.0	0.24 ± 0.06	3.99 ± 1.49	5.7	94.3
[1-^{14}C] 16:0	0.25	0.04 ± 0.01	0.93 ± 0.44	4.2	95.8
	0.50	0.08 ± 0.05	1.98 ± 0.47	3.8	96.2
	1.0	0.14 ± 0.03	3.19 ± 1.20	4.2	95.8
	2.0	0.36 ± 0.18	3.75 ± 0.99	8.8	91.2

All incubations contained 20 shoots (ca. 0.9 g fresh wt) and the tissues were incubated for the times indicated with 17.8 nmoles of the indicated substrate.
[a]Mean ± SD; minimum of 4 experiments.

The amount of label in the chloroform phase also increased with time for all 3 substrates (Table 2). After 0.25 hr, tissues incubated with 18:1 were found to have the largest amount of label associated with the organic phase. By 0.5 hr, comparable amounts of label from 16:0 and 18:1 were associated with the organic phase (1.94 nmol/g fresh wt and 1.98 nmol/g fresh wt, respectively). This was also true for 1 hr and 2 hr incubation times. At all incubation times, however, the relative amount of label in the chloroform fraction from incubations with 18:2 was much lower than that from 18:1 and 16:0.

The percentage of the uptake that was associated with the aqueous phase and organic phase was also calculated for the 3 substrates. The percentage in the aqueous phase was found to increase with time for all substrates (Table 2). Tissues incubated with 18:2 had the greatest increase in percentage of label incorporated into the aqueous phase with time. This increase in percentage was largest between 0.25 hr and 0.5 hr. After 1 hr, the percentage in the aque-

TABLE 3

The Distribution of Label into the Water-Soluble Breakdown Products

Fraction	Aqueous breakdown products	Incorporation (nmol/g fresh wt) Incubation time (hr) 0.25 (4)[a]	0.50 (4)[a]	1.0 (5)[a]	2.0 (12)[a]
Amino acid					
	Glutamate	0.16 ± 0.09[b]	0.16 ± 0.09	0.49 ± 0.18	0.75 ± 0.21
Organic acid					
	Succinate	0.07 ± 0.03	0.14 ± 0.06	0.19 ± 0.03	0.25 ± 0.08
	Malate	–[c]	0.16 ± 0.08	0.21 ± 0.06	0.42 ± 0.14
	Citrate	–	0.02	–	0.04 ± 0.02
	α-Ketoglutarate	–	0.01	–	–
	Oxaloacetate	–	–	0.001	0.03 ± 0.01
	Aconitate	–	0.005	0.008	0.03 ± 0.02
	Malonate	–	–	–	0.06 ± 0.04
Carbohydrate		0.002 ± 0.001	0.010 ± 0.004	0.012 ± 0.009	0.065 ± 0.020

All incubations contained 20 shoots (ca. 0.9 g fresh wt) and the tissues were incubated for the times indicated with 17.8 nmoles of 18:2.

[a]Number of observations for the time indicated.

[b]Mean ± standard deviation.

[c]None detected.

ous phase began to level off, so that by 4 hr, the percentage was only 46% (data not shown). The percentage of label from 18:1 and 16:0 also increased, but in contrast to 18:2, this percentage remained quite low.

Distribution of Label into Water-Soluble Products

To determine the degradative process of 18:2 involved in the production of water-soluble products, the aqueous phase was analyzed for the distribution of label in amino acids, organic acids and carbohydrates. The low level of incorporation into the aqueous phase using 18:1 and 16:0 as substrates made measuring the quantity of label found in the amino acid, organic acid and carbohydrate fractions difficult. Therefore, quantitative analysis of the breakdown products of 18:1 and 16:0 was not carried out.

The distribution of label from 18:2 in the aqueous fractions was determined with increasing time (Table 3). At 0.25 hr, ca. 68% of the label in the aqueous phase was associated with the amino-acid fraction, 31% with the organic acids and 1% with the carbohydrate fraction. With an increase in time, this distribution changed. We observed a decrease in the percentage of label in the amino-acid fraction and an increase in the percentage in the organic and carbohydrate fractions. By 2 hr, ca. 46% of the label was in amino acids, 50% in organic acids and 4% in carbohydrates. At all incubation times, the amount of label in the carbohydrate fraction was minimal and this fraction was not further analyzed.

The individual water-soluble breakdown products were identified by GC. The data presented in Table 3 indicate that, principally, 3 compounds were labeled: glutamic acid, succinic acid and malic acid. Glutamic acid was the only amino acid labeled at all incubation times. The amount of label from 18:2 incorporated into glutamic acid remained essentially constant until 0.5 hr (0.16 nmol/g fresh wt), then rapidly increased to 0.49 nmol/g fresh wt at 1 hr and 0.75 nmol/g fresh wt at 2 hr. In addition, of the individual water-soluble breakdown products, the amount of label in glutamic acid was found to be the highest at all incubation times.

Succinic and malic acids were the principal organic acids labeled. At 0.25 hr, the label was associated only with succinic acid, but by 0.5 hr, both succinic and malic acids were labeled. In addition, the quantity of malic acid was slightly higher than that of succinic acid, 0.16 nmol/g fresh wt and 0.14 nmol/g fresh wt, respectively. Whereas the amount of label in both malic and succinic acids increased with time, the amount of label incorporated into malic acid remained higher than the amount in succinic acid. At 2 hr, 0.42 nmol/g fresh wt of the label from 18:2 were incorporated into malic acid, whereas 0.25 nmol/g fresh wt were in succinic acid.

Though malic and succinic acids were the principal organic acids labeled, several other TCA cycle intermediates, e.g., citric and oxalacetic acids, were occasionally observed. They

TABLE 4

The Distribution of Label into Fatty Acids from Indicated Substrates

Substrate	Percentage of incorporation into fatty acids			
	16:0	18:1	18:2	18:3
[1-^{14}C] 18:2	9.3	10.9	70.4	6.6
[1-^{14}C] 18:1	0	67.7	32.3	0
[^{14}C] CO_2	34.3	12.8	39.6	9.7
[1-^{14}C] Acetic acid	18.7	27.9	46.8	6.8

All incubations contained 20 shoots (ca. 0.9 g fresh wt) and the tissue was incubated for 4 hr with 1 μCi of acetic acid, 18:1, or 18:2 or with 100 μCi of CO_2.

were frequently not detected, especially at 0.25 hr and 0.5 hr, and no apparent pattern was found for their appearance.

Where possible, the amino-acid and organic-acid fractions generated by incubations with 16:0 and 18:1 were analyzed. At 2 hr, label was observed only in glutamic acid and succinic acid for incubations with 16:0 as substrate. When 18:1 was substrate, label was detected in glutamic, succinic and malic acids (data not shown).

The appearance of label in malic and succinic acids, along with glutamic acid, indicated the involvement of the TCA cycle. Three lines of evidence argue against the synthesis of TCA cycle intermediates from CO_2 generated by α-oxidation. First, less than 1% of the radioactivity in the aqueous fraction was found in carbohydrates at the shortest incubation time, and this percentage increased with time. As CO_2 fixation enters carbohydrate metabolism initially, this was opposite to the expected trend. Second, a parallel set of incubations carried out in the dark for 0.25-2 hr, which should have diminished CO_2 fixation, did not alter the labeling of the organic and amino acids (data not shown). Finally, tissue incubated with $^{14}CO_2$, [1-^{14}C] acetate, [^{14}C] 18:1 and [^{14}C] 18:2 incorporated label into fatty acids in a manner consistent with β-oxidation of 18:2 (Table 4). The label from 18:2 appeared in 16:0 and 18:1, indicating breakdown and resynthesis. The 16:0/18:0 ratio was 0.85, which was similar to this same ratio when acetate (0.67) was the substrate, but quite different from the ratio obtained when CO_2 (3.4) was the substrate. Thus, 18:2 was broken down to acetyl-CoA by the β-oxidation pathway. No evidence was found for breakdown by α-oxidation.

Lipoxygenase did not appear to be involved in the degradation of 18:2 in this tissue. Numerous attempts to identify labeled lipids other than palmitic, stearic, oleic and linoleic acids were unsuccessful. Analyses were intended to detect either shorter-chain fatty acids or polar fatty acids. Both polar and nonpolar GC columns in conjunction with temperature programs were used and failed to reveal any possible products of lipoxygenase. Furthermore, whereas 16:0 and 18:1 are not substrates for lipoxygenase, their degradation led to the same products as 18:2, albeit in much lower quantities.

Distribution of Label into Lipids

To determine whether the observed differences in the rate of breakdown between 18:2, 18:1 and 16:0 were related to differences in their incorporation into lipids, the distribution of label from these 3 fatty acid substrates into the major lipid classes was studied. This was accomplished by using silicic acid columns to separate the organic phase into neutral lipid, glycolipid and phospholipid fractions. The results indicated that the label from 18:2, 18:1 and 16:0 was incorporated into similar lipids and that the amount of label incorporated into lipids was comparable for all 3 fatty acids.

At 0.25 hr, the label was principally observed in the neutral lipid fraction for all 3 substrates (Table 5). With an increase in time, the amount of label in the neutral lipid fraction decreased for 18:1. For both 16:0 and 18:2 substrates, the amount of label increased until 1 hr, then leveled off. This increase in label was more rapid for 16:0 than 18:2. For all 3 substrates, the neutral lipid fraction was primarily free fatty acid at all incubation times (data not shown). For each substrate, the free fatty acid was found to be the substrate used in the incubation, and thus represented fatty acid that had been absorbed or transported into the cell but was not yet metabolized. For 16:0, however, although the majority of the label was still free fatty acid, ca. 15% of the label was also observed to be incorporated into several other neutral lipids as early as 0.5 hr. The identities of the lipids were not determined.

The amount of label incorporated into

TABLE 5

The Distribution of Label into the Three Major Lipid Classes

Substrate	Incubation time (hr)	Incorporation (nmol/g fresh wt)		
		Neutral lipids	Glycolipids	Phospholipids
[1-^{14}C] 18:2	0.25	0.38 ± 0.09[a]	0.09 ± 0.03	0.19 ± 0.05
	0.50	0.45 ± 0.12	0.14 ± 0.06	0.30 ± 0.10
	1.0	0.66 ± 0.23	0.12 ± 0.08	0.61 ± 0.16
	2.0	0.72 ± 0.31	0.32 ± 0.11	1.04 ± 0.31
[1-^{14}C] 18:1	0.25	1.99 ± 0.52	0.21 ± 0.09	0.46 ± 0.21
	0.50	1.19 ± 0.43	0.15 ± 0.05	0.60 ± 0.20
	1.0	1.89 ± 0.41	0.16 ± 0.03	1.17 ± 0.47
	2.0	1.50 ± 0.65	0.18 ± 0.03	2.01 ± 0.51
[1-^{14}C] 16:0	0.25	0.73 ± 0.29	0.05 ± 0.02	0.15 ± 0.08
	0.50	1.40 ± 0.57	0.17 ± 0.05	0.41 ± 0.11
	1.0	1.84 ± 0.43	0.13 ± 0.07	1.04 ± 0.23
	2.0	1.84 ± 0.62	0.14 ± 0.06	1.65 ± 0.51

All incubation contained 20 shoots (ca. 0.9 g fresh wt) and the tissues were incubated for the times indicated with 17.8 nmoles of the indicated substrate.

[a]Mean ± SD; minumum of 4 experiments.

glycolipids is relatively low for all 3 substrates. The glycolipids were difficult to identify because the amount of label in this fraction was low for all 3 substrates and the results were often inconsistent. The glycolipids expected to be labeled were monogalactosyldiacylglycerol (MDGD) or digalactosyldiacylglycerol (DGDG). However, TLC analysis showed the major radioactive lipid did not correspond to MGDG or DGDG standards, but migrated ahead of MGDG. Although the glycolipids were not identified, they were the same for all 3 fatty acids. Further, the incorporation of label into glycolipids was minimal for the 3 substrates and could not account for the significant difference in the rate of breakdown between these fatty acids.

At 0.25 hr, the largest amount of label in phospholipids was associated with tissues incubated using 18:1 as substrate (0.46 nmol/g fresh wt). Tissues incubated using 16:0 and 18:2 showed similar molar quantities, 0.15 nmol/g fresh wt and 0.19 nmol/g fresh wt, respectively. The amount of label in the phospholipid fraction increased with time for all 3 substrates. By 2 hr, 1.65 nmol/g fresh wt of the label from 16:0 were incorporated into phospholipids, and 1.04 nmol/g fresh wt and 2.01 nmol/g fresh wt of the label from 18:2 and 18:1 were found incorporated into phospholipids. The individual phospholipids were identified. At all incubation times, the principal phospholipid labeled was phosphatidylcholine (PC), and the label from all 3 fatty acids were incorporated into similar phospholipids.

DISCUSSION

The data presented in this study indicate that the 5-day-old barley tissue has little preference as to which fatty acid substrate enters the tissue. The amount of label and the rate at which the label was taken up were similar for tissues incubated with 18:2, 18:1 and 16:0. Once in the tissue, however, only 18:2 was extensively degraded to water-soluble products. The same products were broken down from 16:0 and 18:1 as 18:2, but at a much slower rate.

The water-soluble breakdown products were identified as amino acids and organic acids, indicating the involvement of the TCA cycle. These results suggest β-oxidation was the oxidative process responsible for the degradation of the 3 fatty acids. In addition, we observed that label from 18:2 was reincorporated into fatty acids. These observations are consistent with the conclusion that β-oxidation was the degradative process involved because the initial product of β-oxidation, acetyl-CoA, may also be a substrate for fatty acid synthesis. In light of the above observations, the product of β-oxidation in leafy tissue would appear to be a substrate primarily for further metabolism in the mitochondria and, to a lesser extent, for fatty acid synthesis in the plastids.

The observed differences in the extent of β-oxidation between 18:2 and the other 2 fatty acid substrates cannot be readily explained by their incorporation into lipids. Label from all 3 fatty acids were found to be incorporated into the same lipids (primarily PC) and the

amounts of label incorporated were not substantially different for the 3 substrates.

The data suggest 2 possible metabolic routes for the fatty acids to follow on entering the tissue. Because both β-oxidation and incorporation of fatty acids into phospholipids require a fatty acyl-CoA as substrate, the observation that all 3 fatty acids were incorporated into phospholipids implies that each of the free fatty acid substrates was activated to the CoA thioester. After a fatty acyl-CoA is formed, the fatty acyl-CoA may be degraded by one of two possible pathways: (a) the fatty acyl-CoA is metabolized to fatty acyl-PC and then the fatty acid moiety (preferentially 18:2) is subsequently broken down by β-oxidation, or (b) the fatty acyl-CoA may directly enter either the β-oxidation sequence or phospholipid synthesis. In the first proposed pathway, the fatty acyl-PC is an intermediate. Because all 3 fatty acid substrates are incorporated into PC in similar amounts, the observed differences in the extent of breakdown would reflect a preferential breakdown of 18:2-PC. If this model is correct, one would expect to observe a rapid turnover in 18:2-PC with an increase in time, because 18:2 is preferentially degraded. Morgan (17) presented evidence that the amount of 18:2-PC does not rapidly turn over compared with 16:0 and 18:1. In pulse-chase experiments, the quantity of 18:2-PC continued to slowly increase during a 4 hr chase, indicating that very little, if any, turnover of the 18:2-PC was occurring. Further, initial incorporation of 18:2 into 18:2-PC before β-oxidation occurs would be surprising in light of how rapidly (<15 min) the degradation products appear. These results indicate that fatty acyl-PC is probably not involved in the degradation sequence. Thus, the other model, i.e., β-oxidation independent of incorporation into lipids, is probably the likely route.

The data presented indicates that the product of β-oxidation, acetyl-CoA, is incorporated into amino and organic acids as well as fatty acids in leafy tissue. Further, a preferential breakdown of 18:2 over 16:0 and 18:1 occurs. This may have significant implications in the metabolic role of β-oxidation in green plants.

REFERENCES

1. Goodwin, T.W., and Mercer, E.I. (1983) in Introduction to Plant Biochemistry (Goodwin, T.W., and Mercer, E.I., eds.), pp. 213-219, Pergamon Press, NY.
2. Stumpf, P.K. (1980) The Biochemistry of Plants, Vol. 4, pp. 85-204, Academic Press, NY.
3. Hitchcock, C., and James, A.T. (1964) J. Lipid Res. 5, 593-599.
4. Galliard, T. and Mercer, E.I. (1975) in Recent Advances in the Chemistry and Biochemistry of Plant Lipids (Galliard, T., and Mercer, E.I., eds) pp. 332-348, Academic Press, NY.
5. Hitchcock, C., and Nichols, B.W. (1971) in Plant Lipid Biochemistry (Hitchcock, C., and Nichols, B.W., eds.), pp. 213-217, Academic Press, NY.
6. Stumpf, P.K., and Barber, G.A. (1956) Plant Physiol. 31, 304-308.
7. Rebeiz, C.A., and Castelfranco, P. (1964) Plant Physiol. 39, 932-936.
8. Yamada, M., and Stumpf, P.K. (1965) Plant Physiol. 40, 653-664.
9. Cooper, T.G., and Beevers, H. (1969) J. Biol. Chem. 244, 3507-3513.
10. Cooper, T.G., and Beevers, H. (1969) J. Biol. Chem. 244, 3514-3520.
11. Gerhardt, B. (1981) FEBS Lett. 126, 71-73.
12. Macey, M., and Stumpf, P.K. (1983) Plant Sci. Letter. 28, 207-212.
13. Breidenbach, R.W., Kahn, A., and Beevers, H. (1968) Plant Physiol. 43, 705-713.
14. Stumpf, D.K., and Burris, R.H. (1979) Anal. Biochem. 95, 311-315.
15. Gehrke, C.W., and Leimer, K. (1971) J. Chromatogr. 57, 219-238.
16. Mohr, W.P., and Cocking, E.C. (1968) J. Ultrastruct. Res. 21, 171-181.
17. Morgan, M.S. (1981) Linolenic Acid Biosynthesis in Green Plants, Ph.D. Dissertation, Miami University, Oxford, OH.

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Effects of Bile Acid Oxazolines on Gallstone Formation in Prairie Dogs

BERTRAM I. COHEN,* ANIL K. SINGHAL, RICHARD J. STENGER, PATRICIA MAY-DONATH, JUDITH FINVER-SADOWSKY, CHARLES K. MCSHERRY and **ERWIN H. MOSBACH,** *Departments of Surgery and Pathology, Beth Israel Medical Center and Mount Sinai School of Medicine, 10 Nathan D. Perlman Place, New York, NY 10003*

ABSTRACT

The effects of 2 bile acid analogs, chenodeoxy-oxazoline [2-(3α,7α-dihydroxy-24-nor-5β-cholanyl)-4,4-dimethyl-2-oxazoline] and ursodeoxy-oxazoline [2-(3α,7β-dihydroxy-24-nor-5β-cholanyl)-4,4-dimethyl-2-oxazoline] were examined in the prairie dog model of cholesterol cholelithiasis. Gallstones and biliary cholesterol crystals were induced in 5 out of 6 male prairie dogs fed a semisynthetic diet containing 0.4% cholesterol for 8 weeks. Six animals maintained on a low cholesterol control diet (0.08% cholesterol) exhibited neither gallstones nor biliary cholesterol crystals. The addition of 0.06% chenodeoxy-oxazoline to the lithogenic diet did not prevent induced cholelithiasis or the appearance of cholesterol crystals in bile. In contrast, 0.06% dietary ursodeoxy-oxazoline prevented gallstones in 5 out of 6 prairie dogs (but cholesterol crystals were present in the bile of 4 of these animals). Histologically, most of the livers from the prairie dogs fed the cholesterol-supplemented semisynthetic diet showed bile duct proliferation, inflammatory infiltration and fibrosis along the portal tracts. These pathologic changes were generally not ameliorated by adding chenodeoxy-oxazoline or chenodeoxy-oxazoline plus chenodeoxycholic acid to the diet. Portal tract pathology was markedly reduced in most animals by adding ursodeoxy-oxazoline to the cholesterol-supplemented diet. The pathologic changes overall could best be correlated with the presence of gallstones, but not with the incidence of biliary cholesterol crystals.
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INTRODUCTION

In the developed countries, cholesterol is the major component of most gallstones (1-4). The etiology of cholesterol gallstone disease is related to the relative stability of cholesterol-containing micelles or liquid crystals in the bile: if the bile is supersaturated with cholesterol, crystals of cholesterol monohydrate precipitate and eventually aggregate to form gallstones (3, 5,6). The 3,7-dihydroxy bile acids, chenodeoxycholic acid and ursodeoxycholic acid, have been used clinically to dissolve gallstones (7-10). These bile acids desaturate the bile by decreasing the amount of cholesterol in relation to the other 2 biliary lipids, bile acids and lecithin, and, in selected patients, dissolution of gallstones occurs during a period of 6-24 mo (10-12). Observations have recently been made that some of the so-called hydrophilic bile acids, e.g., ursodeoxycholic acid, may have a dual-action mechanism. They not only favor the desaturation of bile but also prevent the transition of a liquid-crystalline phase, consisting of cholesterol and lecithin, to crystalline cholesterol monohydrate (13). Bile acid therapy of cholesterol cholelithiasis suffers from a number of disadvantages: (a) a requirement for prolonged, daily administration of the drug; (b) the relatively large dosage of bile acid required, ca. 1-1.5 g/day; (c) loss of active drug from the enterohepatic circulation via excretion and bacterial 7-dehydroxylation to form the potentially hepatoxic lithocholic acid (14). Thus, new drugs with greater efficacy and biological stability need to be developed and tested.

Our laboratory employs the prairie dog model of cholesterol cholelithiasis with the goal of developing more effective cholelitholytic drugs. In the present study, we tested 2 bile acid analogs modified at C24 of the side chain, namely, chenodeoxy-oxazoline and ursodeoxy-oxazoline, because previous work had shown that these compounds had an increased resistance to bacterial 7-dehydroxylation (15). In addition, the bile acid oxazolines are known to inhibit the growth of those intestinal anaerobic bacteria that degrade chenodeoxycholic acid and ursodeoxycholic acid to lithocholic acid (15). We tested the ability of the bile acid oxazolines to prevent cholesterol-induced cholelithiasis and measured their effect on biliary lipids, tissue cholesterol and hepatic morphology.

MATERIALS AND METHODS

Animals and Diet

Adult male prairie dogs (*Cynomys ludovicianus,* trapped in the wild) were purchased

*To whom correspondence should be addressed.

from Fur and Feather Game Farm, Green Bay, WI. The animals were quarantined for 2 weeks and fed Purina rat chow and water ad libitum. The prairie dogs were maintained according to the guidelines developed and administered by the Animal Facility of Beth Israel Medical Center. The animals were then weighed and divided into 5 groups: group 1, semisynthetic diet (0.08% cholesterol, Teklad, Madison, WI); group 2, semisynthetic diet plus 0.4% cholesterol; group 3, semisynthetic diet plus 0.4% cholesterol plus 0.06% 2-(3α,7α-dihydroxy-24-nor-5β-cholanyl)-4,4-dimethyl-2-oxazoline (chenodeoxy-oxazoline); group 4, semisynthetic diet plus 0.4% cholesterol plus 0.06% 2-(3α,7β-dihydroxy-24-nor-5β-cholanyl)-4,4-dimethyl-2-oxazoline (ursodeoxy-oxazoline); group 5, semisynthetic diet plus 0.4% cholesterol plus 0.03% chenodeoxycholic acid plus 0.06% chenodeoxy-oxazoline. The semisynthetic diet contained (g/kg): soy protein, 202.3; sucrose, 565.7; corn starch, 139.0; corn oil, 16.2; fiber (cellulose), 25.8; mineral mix (Teklad 17082), 40.0; vitamin mix (Teklad 40060), 10.0; flavor (sessalom), 1.0. The cholesterol-enriched diets contained 0.2% cholesterol from egg yolk powder and 0.2% cholesterol from reagent-grade cholesterol (Sigma Chemical Co., St. Louis, MO). The animals were fed the pelleted experimental diets for 8 weeks, except that they were starved for 24 hr before sacrifice. At the end of the experiment (8 weeks), the animals were anesthetized with 100 mg of ketamine hydrochloride (Bristol Labs, Syracuse, NY) and 20 mg of xylazine (Haver-Lockhard, Shawnee, KA). The gallbladder of each animal was excised, opened and the gallbladder bile examined under a polarizing microscope for cholesterol crystals and gallstones. Blood was obtained by cardiac puncture to determine plasma cholesterol. The liver was excised and weighed and portions were taken to determine cholesterol concentration and for histologic study.

Reference Compounds

Chenodeoxycholic acid (Canada Packers, Inc., Ontario, Canada) and ursodeoxycholic acid (Tokyo Tanabe Co., Tokyo, Japan) were used to synthesize chenodeoxy-oxazoline and ursodeoxy-oxazoline, respectively, using methods already described (16,17). Chenodeoxycholic acid and ursodeoxycholic acid were analyzed for their methyl ester trimethylsilylether derivatives by gas liquid chromatography (GLC) on 1% HiEff 8BP and found to be greater than 99.5% pure.

5α-Cholestane (Applied Science, State College, PA) was used as an internal standard for the GLC determinations of cholesterol. 3α,7α-Dihydroxy-12-keto-5β-cholanoic acid was used as a recovery standard to analyze biliary bile acids (18-21).

GLC and GLC with Mass Spectroscopy (MS)

GLC of liver, plasma and bile cholesterol was carried out on a Hewlett-Packard 5830 gas chromatograph using conditions already described (18). Bile samples containing the oxazolines were hydrolyzed to give the free bile acids and analyzed (21). Biliary bile acids were analyzed for their methyl ester acetates on 0.5% OV-210 (21). Identification of steroids in bile was carried out on a Hewlett-Packard 5992B mass spectrometer using the following conditions: 3 ft glass column, 4 mm o.d., 2 mm i.d., packed with 3% SP-2250 on 100/120 Supelcoport; gas chromatograph—oven temp 260 C, injector temp 265 C, mass spectrometer —pressure 2×10^{-6} torr, source temp 140 C.

Determination of Lipids in Liver, Plasma and Bile

The determination of cholesterol in liver and plasma was carried out as described earlier (18). Gallbladder bile obtained at sacrifice was centrifuged at 2000 × *g* for 10 min, and aliquots of the supernatant solution were prepared to determine biliary lipids (21). The bile samples of the animals fed the oxazolines were analyzed by thin layer chromatography (TLC) (silica gel using benzene/acetone, 70:30, v/v). The regions corresponding to the oxazolines were devoid of these compounds. In addition, extracting the bile with ethyl acetate followed by GLC also showed no oxazolines. Consequently, the bile samples were hydrolyzed in the standard manner (21). The phospholipids were analyzed by a modification of the procedure of Fiske and Subbarow (21).

Calculations and Statistics

The lithogenic indices of the bile were determined as described by Carey (22). All data are reported as mean ± SEM. Student's *t* test or chi square was used to determine statistical significance (23). Analysis of variance was used for multiple comparisons.

Histology

Thin slices of each liver were fixed in Millonig's buffered formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin, periodic acid-Schiff, trichrome and reticulin stains.

RESULTS

Male adult prairie dogs were fed the experimental diets for 8 weeks. The animals showed no adverse effects to the diets and no animals died during the experiment. The average initial and final weights among groups did not differ significantly (Table 1); daily food intake and fecal outputs were also similar.

At sacrifice, gallbladders were removed and opened to determine the presence of gallstones. Bile was analyzed under a polarizing microscope to determine the presence of cholesterol crystals. These data are summarized in Table 2. Animals fed the semisynthetic diet (group 1) had no cholesterol crystals or gallstones. In the group fed the semisynthetic diet plus 0.4% cholesterol, 5 out of 6 animals had gallstones and cholesterol crystals. Feeding the bile acid analog, chenodeoxy-oxazoline, failed to prevent gallstones or crystals; 4 out 5 animals had gallstones as well as cholesterol crystals. Interestingly, when chenodeoxy-oxazoline was combined with chenodeoxycholic acid, the incidence of cholesterol gallstones was reduced to 40% but cholesterol crystals were present in 4 out of 5 animals. Ursodeoxy-oxazoline was the most effective bile acid analog in preventing cholesterol gallstones (1 out of 6 animals had gallstones); however, cholesterol crystals were present in the bile of 4 out of 6 animals.

Cholesterol concentrations in liver, plasma and bile are summarized in Table 3. Both ursodeoxy-oxazoline and the combination of chenodeoxycholic acid plus chenodeoxy-oxazoline significantly lowered liver and plasma cholesterol levels. However, neither of these diets was able to significantly reduce biliary cholesterol. As in previous studies, animals fed the nonlithogenic diet had the lowest cholesterol levels (24).

Biliary lipid composition is summarized in Table 4. Biliary cholesterol was elevated in all groups fed cholesterol compared with controls. In conjunction with these increases, the lithogenic indices in all of these groups were above 1.0. Biliary bile acid concentrations were not statistically different; however, phospholipid concentrations were lower in the animals fed chenodeoxy-oxazoline compared with the other groups.

Analyses of the biliary bile acids revealed several differences between the groups. In the

TABLE 1

Weights, Food Intakes and Fecal Outputs of Prairie Dogs[a]

Group number	Number of animals	Diet	Initial weight (g)	Final weight (g)	Food intake (g/day)	Fecal output (g/day)
1	6	Semisynthetic diet (SSD)	993	1188	30.7	1.43
2	6	SSD + 0.4% cholesterol (CH)	973	1123	33.9	1.30
3	5	SSD + 0.4% CH + 0.06% chenodeoxy-oxazoline	941	1183	27.6	2.02
4	6	SSD + 0.4% CH + 0.06% ursodeoxy-oxazoline	862	1092	35.0	1.40
5	5	SSD + 0.4% CH + 0.03% chenodeoxycholic acid + 0.06% chenodeoxy-oxazoline	768	1124	40.0	1.42

[a]Numbers are means for each group.

TABLE 2

Incidence of Cholesterol Gallstones and Cholesterol Crystals in Prairie Dogs

Group number	Number of animals	Diet	Incidence of Cholesterol gallstones	Incidence of Cholesterol crystals
1	6	Semisynthetic diet (SSD)	0/6	0/6
2	6	SSD + 0.4% cholesterol (CH)	5/6[a]	5/6[a]
3	5	SSD + 0.4% CH + 0.06% chenodeoxy-oxazoline	4/5[a]	4/5[a]
4	6	SSD + 0.4% CH + 0.06% ursodeoxy-oxazoline	1/6[b]	4/6[a]
5	5	SSD + 0.4% CH + 0.03% chenodeoxycholic acid + 0.06% chenodeoxy-oxazoline	2/5	4/5[a]

[a]Differs from group 1 by chi square test, $p<0.01$.
[b]Differs from group 2 by chi square test, $p<0.05$.

TABLE 3

Cholesterol Concentrations in the Prairie Dog at Week 8[a]

Group number	Number of animals	Diet	Liver cholesterol (mg/g)	Plasma cholesterol (mg/dl)	Bile cholesterol (mg/ml)
1	6	Semisynthetic diet (SSD)	2.87 ± 0.28	145 ± 16	0.76 ± 0.28
2	6	SSD + 0.4% cholesterol (CH)	5.74[b] ± 0.88	534[b] ± 77	3.87[b] ± 0.49
3	5	SSD + 0.4% CH + 0.06% chenodeoxy-oxazoline	4.73[b] ± 0.26	337[b,d] ± 13	4.54[b] ± 0.76
4	6	SSD + 0.4% CH + 0.06% ursodeoxy-oxazoline	3.31[c] ± 0.30	269[d] ± 34	3.43[b] ± 0.52
5	5	SSD + 0.4% CH + 0.03% chenodeoxycholic acid + 0.06% chenodeoxy-oxazoline	4.11[b] ± 0.31	340[e] ± 47	3.16 ± 0.82

[a]Numbers are means ± SEM.
[b]Differs from group 1, $p<0.01$.
[c]Differs from group 3, $p<0.01$.
[d]Differs from group 2, $p<0.01$.
[e]Differs from group 2, $p<0.02$.

TABLE 4

Lipid Composition and Lithogenic Index of Gallbladder Bile[a]

Group number	Number of animals	Diet	Cholesterol (mole %)	Phospholipids (mole %)	Bile acids (mole %)	Lithogenic index
1	6	Semisynthetic diet (SSD)	1.4 ± 0.4	7.6 ± 0.4	91.0 ± 0.7	0.42 ± 0.08
2	6	SSD + 0.4% cholesterol (CH)	7.7[b] ± 0.7	12.9 ± 1.3	79.4 ± 1.8	1.86[b] ± 0.32
3	5	SSD + 0.4% CH + 0.06% chenodeoxy-oxazoline	7.9[b] ± 1.8	3.2[b] ± 0.8	88.9 ± 2.5	3.03[b] ± 0.60
4	6	SSD + 0.4% CH + 0.06% ursodeoxy-oxazoline	5.5[b] ± 0.2	9.8[c] ± 2.4	82.7 ± 2.5	1.46[b,e] ± 0.08
5	5	SSD + 0.4% CH + 0.03% chenodeoxycholic acid + 0.06% chenodeoxy-oxazoline	5.5[b] ± 0.5	2.7[d] ± 0.5	91.8 ± 0.2	2.27 ± 0.14

[a]Numbers are means ± SEM; the lithogenic index was calculated as described by Carey.
[b]Differs from group 1, $p<0.01$.
[c]Differs from group 3 and 5, $p<0.01$.
[d]Differs from group 1, 2 and 4, $p<0.01$.
[e]Differs from group 3, $p<0.01$.

animals fed chenodeoxy-oxazoline or chenodeoxycholic acid plus chenodeoxy-oxazoline, chenodeoxycholic acid became the predominant biliary bile acid (Table 5). No ursodeoxycholic acid was present in the bile. When ursodeoxy-oxazoline was in the diet, ursodeoxycholic acid comprised 29.8% of the biliary bile acids with chenodeoxycholic acid comprising an additional 37.7%. Only small amounts of lithocholic acid and deoxycholic acid were present.

Histologic sections of the livers from all animals fed the semisynthetic diet supplemented with 0.4% cholesterol revealed a variable amount of micro- and macrovacuolar lipid accumulation in the parenchyma, which was otherwise unaltered. Specifically, no morphologic evidence was found of bile stasis in any of the prairie dogs. Bile duct proliferation, inflammatory infiltration and fibrosis were evident in the portal tracts in 4 of 6 animals fed the semisynthetic diet supplemented with cholesterol alone; in 4 of 5 prairie dogs given the semisynthetic diet supplemented with cholesterol and chenodeoxy-oxazoline; in 2 of 5 animals fed the semisynthetic diet supplemented with cholesterol, chenodeoxy-oxazoline, and chenodeoxycholic acid; and in 1 of 6 prairie dogs given the semisynthetic diet supplemented with cholesterol and ursodeoxy-oxazoline. The portal tract abnormalities were most striking in the group fed the basic diet plus cholesterol (Fig. 1) and in the group given the basic diet plus cholesterol and chenodeoxy-oxazoline (Fig. 2). Portal tract changes were minimal in most of the animals given the basic diet plus cholesterol and ursodeoxy-oxazoline (Fig. 3).

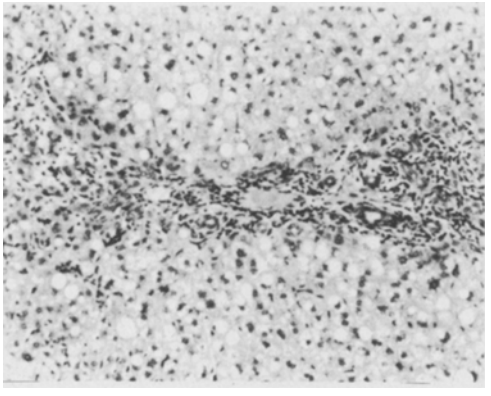

FIG. 1. Liver from a prairie dog fed a semisynthetic diet supplemented with cholesterol. Across the center of the microscopic field is an expanded portal tract displaying bile duct proliferation, heavy inflammatory infiltration and mild reactive fibrosis. The adjacent parenchyma shows mild micro- and macrovacuolar steatosis (H&E stain, X 120).

TABLE 5

Biliary Bile Acid Composition in Prairie Dog[a]

Group number	Number of animals	Diet	Composition, percentage by weight			
			LA	DA	CDA	UDA	CA
1	6	Semisynthetic diet (SSD)	0.3 ± 0.2	5.1 ± 0.9	15.3 ± 1.6	—	79.3 ± 3.6
2	6	SSD + 0.4% cholesterol (CH)	0.9 ± 0.4	1.1 ± 0.5	57.9[b] ± 3.4	—	40.1[b] ± 2.9
3	5	SSD + 0.4% CH + 0.06% chenodeoxy-oxazoline	2.3 ± 0.5	0.8 ± 0.4	88.7[b,c] ± 2.2	—	8.0[b,c] ± 1.0
4	6	SSD + 0.4% CH + 0.06% ursodeoxy-oxazoline	2.8 ± 0.5	1.9 ± 0.5	37.7[b,c] ± 1.1	29.8 ± 1.7	20.4[b,c] ± 1.8
5	5	SSD + 0.4% CH + 0.03% chenodeoxycholic acid + 0.06% chenodeoxy-oxazoline	0.6 ± 0.1	—	88.4 ± 1.3	—	11.0[b,c] ± 1.3

The abbreviations used are: LA, lithocholic acid; DA, deoxycholic acid; CDA, chenodeoxycholic acid; UDA, ursodeoxycholic acid; CA, cholic acid.
[a]Numbers are means ± SEM.
[b]Differs from group 1, $p<0.01$.
[c]Differs from group 2, $p<0.01$.

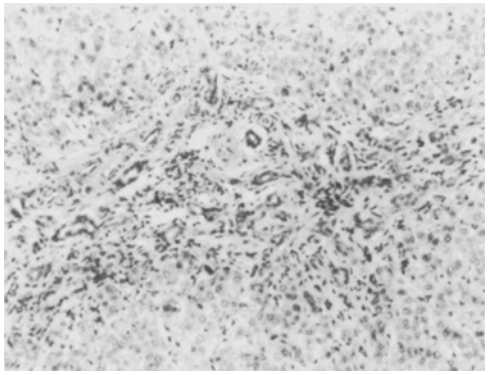

FIG. 2. Liver from a prairie dog fed a semisynthetic diet supplemented with cholesterol and chenodeoxy-oxazoline. Across the center of the field is a greatly expanded portal tract with prominent duct proliferation, moderate inflammatory infiltration and moderate reactive fibrosis. The contiguous parenchyma shows only focal microvacuolar steatosis (H&E stain, X 120).

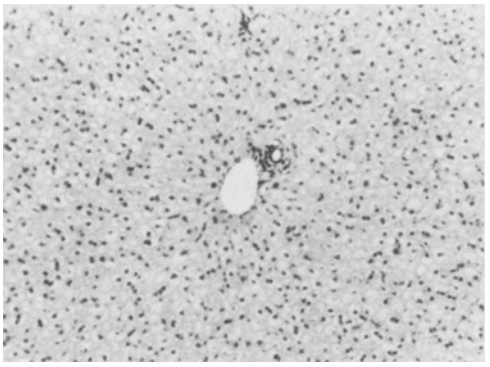

FIG. 3. Liver from a prairie dog fed a semisynthetic diet supplemented with cholesterol and ursodeoxy-oxazoline. The portal tract reveals minimal mononuclear inflammatory infiltration, but is otherwise intact. The contiguous parenchyma exhibits slight focal microvacuolar steatosis, but is essentially unavailable (H&E stain, X 120).

DISCUSSION

This study evaluated the effectiveness of chenodeoxy-oxazoline and ursodeoxy-oxazoline as gallstone-preventing agents in the prairie dog model of cholelithiasis. In previous studies, feeding the free bile acid (chenodeoxycholic acid) to prairie dogs on a lithogenic regimen gave varying results: in one study gallstones and cholesterol crystals were completely absent (25), whereas in another study both gallstones and cholesterol crystals were prominent in the gallbladder (24). The reasons for this discrepancy were not clear. One possibility was the heterogeneity of the prairie dogs used; another was that the amounts of bile acid ingested were lower in our studies than in those of Doty et al. (25). Consequently, a search for more effective gallstone-dissolving agents using this model seemed appropriate.

The prairie dogs used in the present study showed no overt signs of illness over the 8-week experiment. The animals in each group gained weight, ate the dietary regimens and had no diarrhea. Ursodeoxy-oxazoline proved effective in preventing gallstones in 5 out of 6 animals. In 3 of the 5 animals, cholesterol crystals were present in the bile but no stones formed. In an earlier study, we observed that ursodeoxycholic acid (0.03%) prevented gallstones in 5 out of 6 animals, whereas ursodeoxycholic acid at a high dose (0.06%) prevented stones in only 2 out of 6 animals (24). The mechanism whereby ursodeoxy-oxazoline and ursodeoxycholic acid reduced gallstone incidence was probably similar and will be discussed later. In contrast to ursodeoxy-oxazoline, chenodeoxy-oxazoline did not prevent either gallstones or crystals. The animals given this drug had the highest lithogenic index (average of 3.03 ± 0.60) compared with ursodeoxy-oxazoline (1.46 ± 0.08). Recent studies by Salvioli et al. (13) suggested that gallstones could be absent from bile even when the lithogenic index was greater than 1.0 (e.g., 1.46, supersaturated bile). In addition, Fromm et al. have shown that ursodeoxycholic acid can induce gallstone dissolution in spite of the persistence of a lithogenic index above 1 (10). These biles probably contained aggregates of cholesterol and lecithin in the liquid crystalline state because bile acids with hydrophilic properties, e.g., ursodeoxycholic acid, prevent the phase transition of these liquid crystals to microcrystals. Ursodeoxy-oxazoline and ursodeoxycholic acid may prevent gallstones by this type of mechanism, but further studies are needed to verify this point. Though chenodeoxy-oxazoline was ineffective in preventing gallstones, a combination of chenodeoxycholic acid plus chenodeoxy-oxazoline prevented gallstones in 3 out of 5 animals. This combination of drugs did not prevent cholesterol crystals in bile. The combination drug therapy was administered with the expectation that the oxazoline would prevent the bacterial 7-dehydroxylation of chenodeoxycholic acid and this might elevate chenodeoxycholic acid concentration in bile. However, the dose of chenodeoxy-oxazoline reaching the large bowel was apparently too low to achieve this result; fecal steroid analyses showed that in all treated groups lithocholic acid, the 7-dehydroxylation product

of chenodeoxycholic acid, was the major fecal bile acid.

Histologic study of the livers revealed striking changes in the portal tracts of most prairie dogs fed the semisynthetic diet supplemented with cholesterol alone and in most animals fed the semisynthetic diet supplemented with cholesterol and chenodeoxy-oxazoline. The portal tract abnormalities included bile duct proliferation, inflammatory infiltration and reactive fibrosis (Figs. 1 and 2). These pathologic changes were less consistent in the prairie dogs fed the semisynthetic diet supplemented with cholesterol, chenodeoxy-oxazoline and chenodeoxycholic acid, and only 1 of 6 animals fed the semisynthetic diet supplemented with ursodeoxy-oxazoline displayed equivalent portal tract pathology. These morphologic observations can be best correlated with the presence of gallstones. In fact, in the groups with a low incidence of portal tract pathology, the histologic abnormalities were prominent only in those animals with demonstrable gallstones. No similar correlation was found, however, with the presence of biliary cholesterol crystals, because many of the prairie dogs with such crystals revealed little or no portal tract pathology.

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REFERENCES

1. Ilias, A.M., Connor, W.E., Cory, H.T., Lin, D.S., Daves, G.D., Jr., and Krippaehne, W.E. (1980) Gastroenterology 79, 539-544.
2. Nakayama, F. (1966) Clin. Chim. Acta 14, 171-176.
3. Shaffer, E.A. (1980) Canad. J. Surg. 23, 517-532.
4. Sutor, D.J., and Wooley, S.E. (1971) Gut 12, 55-64.
5. Admirand, W.H., and Small, D.M. (1968) J. Clin. Invest. 47, 1043-1052.
6. Carey, M.C., and Small, D.M. (1978) J. Clin. Invest. 61, 998-1026.
7. Bachrach, W.H., and Hofmann, A.F. (1982) Dig. Dis. Sci. 27, 737-761; 833-886.
8. Bell, G.D., Whitney, B., and Dowling, R.H. (1972) Lancet, 1213-1216.
9. Thistle, J.L., Hofmann, A.F., Ott, B.J., and Stephen, D.H. (1978) J. Am. Med. Assn. 239, 1041-1046.
10. Fromm, H., Roat, J.W., Gonzalez, V., Sarva, R.P., and Farivar, S. (1983) Gastroenterology 85, 1257-1264.
11. Hofmann, A.F., Thistle, J.L., Klein, P.D., Szczepanik, P.A., and Yu, P.Y.S. (1978) J. Am. Med. Assn. 239, 1138-1144.
12. Schoenfield, L.J., and Lachin, J.M. (1981)) Ann. Intern. Med. 95, 257-282.
13. Salvioli, G., Igimi, H., and Carey, M.C. (1983) J. Lipid Res. 24, 701-720.
14. Fischer, C.D., Cooper, N.S., Rothschild, M.A., and Mosbach, E.H. (1974) Am. J. Dig. Dis. 19, 877-886.
15. Hylemon, P.B., Fricke, R.J., and Mosbach, E.H. (1982) Steroids 40, 347-357.
16. Ayengar, N.K.N., Singhal, A.K., McSherry, C.K., and Mosbach, E.H. (1981) Steroids 38, 333-345.
17. Cohen, B.I., May, P.S., McSherry, C.K., and Mosbach, E.H. (1982) Steroids 40:701-711.
18. Cohen, B.I., Raicht, R.F., and Mosbach, E.H. (1977) J. Lipid Res. 18, 223-231.
19. Cohen, B.I., Raicht, R.F., Salen, G., and Mosbach, E.H. (1975) Anal. Biochem. 64, 567-577.
20. Cohen, B.I., Raicht, R.F., and Mosbach, E.H. (1974) Lipids 9, 1024-1029.
21. McSherry, C.K., Deitrick, J.E., May, P.S., Niemann, W., Morrissey, K.P., Palmer, R.H., and Glenn, F. (1977) Surg. Gynecol. Obstet. 144, 727-733.
22. Carey, M.C. (1978) J. Lipid Res. 19, 945-955.
23. Snedecor, G.W. (1948) Statistical Methods, pp. 54-81; 182-184, Iowa State College Press, Ames, Iowa.
24. Cohen, B.I., Singhal, A.K., Stenger, R.J., May-Donath, P., Finver-Sadowsky, J., McSherry, C.K., and Mosbach, E.H. (1984) Hepatology 4, 300-307.
25. Doty, J.E., DenBesten, L., Roslyn, J.J., Pitt, H.A., Kuchenbecker, S.L., and Porter-Fink, V. (1982) Am. J. Surg. 143, 48-54.

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Effects of a (n-3) Polyunsaturated Fatty Acid-Deficient Diet on Profiles of Serum Vitellogenin and Lipoprotein in Vitellogenic Trout (*Salmo gairdneri*)

L. FRÉMONT, C. LÉGER, B. PETRIDOU and **M.T. GOZZELINO**, *Station de Recherches de Nutrition, C.N.R.Z.-I.N.R.A., 78350-Jouy-en-Josas, France*

ABSTRACT

During the 6 months of vitellogenesis, 3-year-old female trout *(Salmo gairdneri)* were fed either an enriched (E) or an (n-3) polyunsaturated fatty acid (PUFA)-deficient (D) diet; serum vitellogenin (VG) and lipoproteins ($d < 1.21$ g/ml) were analyzed at the third month of vitellogenesis (September) and at ovulation (December). The serum content of high density lipoproteins (HDL), the major protein class, maintained a mean value of 1500 mg/dl at both stages and with both diets. On the contrary, very low density lipoproteins (VLDL) were 90% higher during vitellogenesis than at spawning time, whereas excess vitellogenin circulated at this period (6580 mg/dl serum with diet E). The diet deficient in (n-3) lowered serum vitellogenin content by 16% in September and by 26% in December. The degree of (n-3) PUFA incorporation moderately decreased in low density lipoproteins (LDL) and in HDL with the (n-3)-deficient diet. The effect was more pronounced for 20:5. On the other hand, essential 22:6 was incorporated into vitellogenin at the same rate in September as in December with diet E (23% and 25%, respectively), whereas after a 3-month deficiency, the percentage fell to 12%; this percentage rose again to 19% at spawning time. These findings show that, although stored (n-3) PUFA were not exhausted after a 6-month dietary deficiency, the incorporation of essential fatty acids (EFA) into vitellogenin during the early stages of oogenesis was low, suggesting changes in egg composition that may influence hatching.
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INTRODUCTION

During primary vitellogenic stages in trout (February to July), very small amounts of vitellogenin (VG), the proteolipid precursor of egg yolk, are incorporated into oocytes and the serum concentrations remain below 20 μg/ml (1). Yolk accumulation really begins at the following stage, which is called exogenous vitellogenesis and corresponds to the uptake of yolk protein and lipids by oocytes as they grow from 0.6 mm to 4.5 mm (2). Lipids ingested during this period can be predicted to influence the fatty acid composition of egg lipids and, thus, the precursors that are carried in the serum.

In a previous study (3), a group of female trout were fed a diet deficient in (n-3) during the final 3 months of vitellogenesis, corresponding to four-fifths of oocyte growth. This period lasted from September to December. Analysis of serum and egg lipids at spawning showed a net decrease in total (n-3) fatty acids compared with a control group receiving cod-liver oil as the dietary lipid source. In the group deficient in (n-3), 22:6 was the major polyunsaturated fatty acid (PUFA) found in blood and eggs.

These results suggest that the levels of essential fatty acids stored in the tissues before and during the early stages of vitellogenesis were sufficient to provide the required (n-3) PUFA for oocyte development. In terms of weight, egg development is minor during the first 3 months of vitellogenesis. However, this period is physiologically important because oocyte structures are being organized, and essential fatty acids or their derivatives may play a role. Similar events occur in anadromous salmonids, which fast from the onset to the end of a spawning migration lasting 2-3 months or more. At the beginning of this period, the gonads are usually in the early stages of maturation, and nutrients for the formation of sexual tissues are probably taken up from endogenous sources.

We carried out the present experiment to evaluate the effects of a diet deficient in (n-3) given during the whole period of exogenous vitellogenesis. Serum lipids have been compared at the third month of vitellogenesis and at spawning. The distribution of components and levels of (n-3) PUFA were evaluated in vitellogenin and other classes of serum lipoproteins (LP) because all lipid vehicles can be used directly or indirectly for the formation of yolk.

MATERIAL AND METHODS

Animals

Female rainbow trout *(Salmo gairdneri)* were reared at 12-15 C in freshwater tanks. They were fed a commercial diet from the

swimming-up stage to the experimental period. Dietary lipid accounted for 8-10% of the dry matter to provide the levels (1% of the diet) of (n-3) PUFA essential to the trout (4,5). The main (n-3) PUFA (in percentages of total fatty acids) were 18:3 (4-5%), 20:5 (1-2%) and 22:6 (2-3%).

In 1980, the trout were 3 years old and had begun the second reproductive cycle. On June 2 they were divided into 2 groups of 19 fish, each fish weighing 1000-1200 g. After a starvation period of 15 days, the experimental diets, either enriched (E) with (n-3) or deficient (D) in (n-3), were given to each group. The sources of fatty acids were cod-liver oil in diet E and lard in diet D. The fatty acid composition of those diets were, respectively: saturates–18.7, 47.9; monoenes–46.8, 36.8; (n-6) polyenes–7.1, 12.2; (n-3) polyenes–25.4, 3.1; 20:5–9.4, 0.6; 22:6–10.6, 1.0 (percentages of total fatty acids).

In September, when the eggs were one-fifth developed (3), the fish were randomly divided into 2 subgroups (I and II) of 8-9 fish each (some died during the experiment). Ca. 10 ml of blood per fish was withdrawn from the dorsal caudal artery, and the animals were subsequently maintained in similar experimental conditions until they were killed at spawning in December. Ca. 4 ml of serum per sample of blood was obtained after low-speed centrifugation. Because of the small volumes, sera from all fish in each subgroup were pooled for analysis. In order to obtain baseline values of endogenous lipid, the September groups were bled after 1 week of starvation. This starvation period was reduced to the 2 days following ovulation in the December groups because the ovulated eggs had to be stripped early when ripe. The serum was isolated and stored at −80 C (6) and collected oocytes were stored at −80 C for further analysis.

Analytical Procedures

Classes of LP were isolated by sequential ultracentrifugation at different densities (7,8). Briefly, a rotor 50 Ti was operated at 10 C and 45,000 rpm (145,000 × *g*) in a Beckman L5 50B centrifuge. The density intervals for LP flotation and the run times were: very low density lipoprotein (VLDL), 18 hr (d < 1.015 g/ml); low density lipoprotein (LDL_1), 24 hr (d 1.015-1.063 g/ml); LDL_2, 24 hr (d 1.063-1.085 g/ml); high density lipoprotein (HDL), 40 hr (d 1.085-1.21 g/ml); VG, 40 hr (d 1.21-1.28 g/ml).

Electrophoresis in agarose gel revealed that LP (d < 1.21 g/ml) had greater mobility than VG, which migrated near the origin. The faster bands, LDL_1 and LDL_2 (unresolved), migrated close to HDL (6). Lipids were extracted from total serum and from the isolated LP or VG with chloroform/methanol (2:1, v/v) (9). Triglycerides (TG) and cholesterol were quantified using enzymatic procedures (Biochemica test combination Boehringer). Phospholipids (PL) were estimated by the method of Barlett (10) and protein according to the method of Lowry et al. (11). Lipid classes were fractionated by thin layer chromatography using hexane/ethyl ether/formic acid (120:60:1.5, v/v/v) as the solvent for development. After saponification of eluted spots by an excess of 15% alcoholic KOH at room temperature, methyl esters were prepared and analyzed (8).

Gas chromatography was performed using an open-tubular glass column (0.5 mm i.d. × 50 m) packed with 0.38% free fatty acid phase (FFAP) as the stationary phase (Supelco, Inc., Bellefonte, PA). The temperatures of the column and the injector were 190 C and 230 C. The peaks were identified by calculating the equivalent chain lengths (12).

The pooled sera of each subgroup were assayed separately. The contents of the LP classes were estimated by analyzing their lipid and protein moieties.

VG was evaluated in 2 steps: (a) the complex floating in the density range 1.21-1.28 g/ml was pure but not quantitatively recovered, therefore, it was analyzed to obtain the ratio of protein to lipid; (b) VG lipid content was obtained indirectly from lipid analysis of the bottom fraction remaining after the LP classes (d < 1.21 g/ml) had been isolated. Electrophoresis revealed that this fraction contained serum proteins and VG with practically no other lipids. Finally, VG levels were calculated from both the protein-lipid ratio and the lipid content.

The levels of (n-3) PUFA in esterified lipid of serum were calculated by multiplying the ratio: [(n-3) PUFA (total 20:5 or 22:6)]/(total fatty acids) by the total fatty acid contents of esterified lipids (TG, cholesterol ester [CE] or PL). Total fatty acids were calculated from the serum content (mg/dl) in TG (a), CE (b) and PL (c) and the relative mean weight of fatty acids in these esters: TG fatty acids = a × 0.90; CE fatty acids = b × 0.44; PL fatty acids = c × 0.70.

RESULTS

Lipoprotein Analysis (LP d < 1.21 g/ml)

Figure 1 clearly shows that serum LP levels were almost unmodified by the dietary deficiency in (n-3) PUFA and that HDL was the

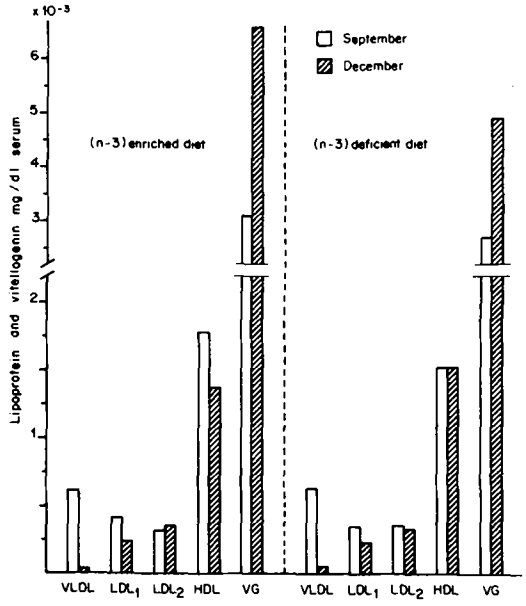

FIG. 1. Total serum contents (mg/dl) of lipoprotein classes (d < 1.21 g/ml) and vitellogenin in female trout. Results are the mean values of the analyses (in duplicate or triplicate) of 2 subgroups of trout per diet. The same subgroups were used in September and December; each included the pooled sera of 8-9 fish.

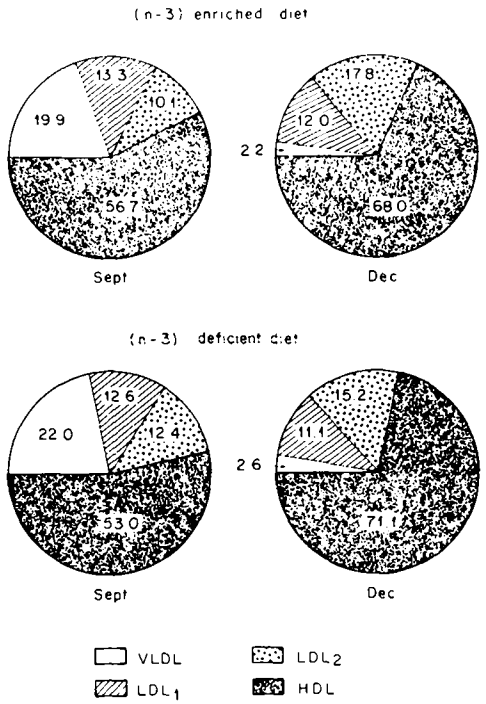

FIG. 2. Mean weight (%) distribution of female trout lipoproteins (d < 1.21 g/ml) in relation to the diet and period of the sexual cycle.

predominant class. The mean value of HDL of the 2 subgroups was 1500 mg/dl at both periods for diet D. A variation between September and December was observed for diet E, but Student's *t* test revealed that results obtained from both samples did not differ significantly (mean-1580).

Total LP levels (mg/dl serum) were not influenced by diet but they were higher in September than in December, 3142 and 2858 compared with 2040 and 2132 for E and D.

As shown in Figure 2, the VLDL class was responsible for the difference, showing a 90% decrease between September and December. The derivate LDL_1 was also affected.

Properties of Vitellogenin

Unlike LP (d < 1.21 g/ml), VG contents changed with the diet and the period in the cycle (Fig. 1). The comparison was performed by analyzing variance. The results show a significant difference between diets and periods. In September and December, the means of the 2 subgroups were 3179 mg/dl and 6580 mg/dl ($P < 0.02$, for diet E, and 2684 and 4876 ($P < 0.05$) for diet D. In addition, the difference between September and December was significantly less ($P < 0.05$) with diet D than with diet E.

The composition of diet did not influence the distribution of the VG components. The lipid and protein percentages in VG for E and D in September and December were, respectively: cholesterol–1.0 throughout; CE–0.5, 0.5, 0.6 and 0.5; TG–5.5, 5.0, 3.0 and 3.0; PL–14.0, 17.0, 14.0 and 14.0; protein–78.5, 76.5, 81.4 and 81.5.

The (n-3) dietary deficiency did not change the protein-lipid ratio, which, however, was influenced by the period in the sexual cycle (3.3 in September and 4.2 in December).

Fatty Acid Distribution

At both seasons, the percentage of 18:1 (n-9) was higher with diet D (18-23%) than with diet E (13-17%), but note that 20:3 (n-9), which is an index of combined (n-6) and (n-3) deficiency (4), was not found in circulating lipids.

Table 1 shows that in the groups with diets enriched with (n-3), the proportion of 20:5 was similar in the diet and the serum (ca. 10%), but circulating lipids were largely enriched in 22:6 compared with the diet. In the groups deficient in (n-3), fatty acid 20:5 fell drastically in serum lipids, whereas 22:6 decreased at a moderate rate.

TABLE 1

Fatty Acid Composition (Percentage of Weight) of Serum LP (d < 1.21 g/ml) and Vitellogenin in Female Trout

Fatty acids	VLDL Sept.	VLDL Dec.	LDL_1 Sept.	LDL_1 Dec.	LDL_2 Sept.	LDL_2 Dec.	HDL Sept.	HDL Dec.	VG Sept.	VG Dec.
Diet E										
Σ Saturates	20.1	25.0	25.6	24.0	26.8	26.1	28.0	26.2	33.1	30.7
Σ Monoenes	35.8	22.9	29.5	25.9	24.6	21.6	23.8	20.8	26.2	22.3
Σ Polyenes	44.1	42.1	45.9	50.1	48.6	52.3	58.2	53.0	40.7	47.0
20:5 n-3	11.1	10.0	9.7	11.4	9.0	10.9	8.9	10.8	6.8	9.1
22:6 n-3	18.4	20.7	22.3	25.8	26.0	29.5	28.6	30.6	23.2	25.4
22:6/20:5	1.6	2.0	2.3	2.2	2.9	2.7	3.2	2.5	3.4	2.9
Diet D										
Σ Saturates	26.0	28.7	32.5	28.1	42.9	28.4	29.2	27.9	43.9	32.6
Σ Monoenes	45.5	36.2	31.7	29.1	23.9	26.3	27.6	25.2	27.1	26.9
Σ Polyenes	28.5	35.1	35.8	42.8	33.2	45.3	43.2	46.9	29.0	40.5
20:5 n-3	1.5	2.0	1.8	2.3	2.0	2.7	2.6	2.9	1.5	2.6
22:6 n-3	9.3	14.0	15.6	18.9	17.2	22.9	22.3	24.0	12.2	19.2
22:6/20:5	6.2	7.0	8.6	8.2	8.6	8.5	8.6	8.3	8.1	7.4

Each result is the mean value of subgroups I and II (see Fig. 1).

Each LP class showed a similar distribution of fatty acids in September and December when (n-3) PUFA were provided to trout. The percentage of 20:5 and especially of 22:6 was slightly higher in December than in September in all LP classes (d < 1.21 g/ml) and, at both seasons, it increased from VLDL to HDL. A similar feature was observed with the diet deficient in (n-3), which, as expected, showed a lower incorporation of (n-3) PUFA. At both seasons, only 1.5-3% of 20:5 remained in all classes of lipoproteins. The essential 22:6 decreased but remained relatively high. The influence of dietary (n-3) deficiency was more pronounced in VLDL and their derivative, LDL_1, and to a lesser degree in LDL_2, than in HDL, which was mainly endogenous.

During vitellogenesis, the influence of diet on the newly synthesized VG was more pronounced than with other LP: percentages of 22:6 were 50% lower in September but only 25% lower in December, when D replaced E.

Figure 3 summarizes the distribution of (n-6) and (n-3) PUFA in VLDL, the class that fluctuated most, and in HDL and VG, the major classes. The lack of dietary (n-3) PUFA caused a decrease in those fatty acids in the serum; this coincided with an increase in (n-6) PUFA, especially 20:4 (n-6) derived from the 18:2 ingested. Finally, the lower levels of 20:5 and 22:6 were partially balanced by an enhancement of 20:4 (n-6).

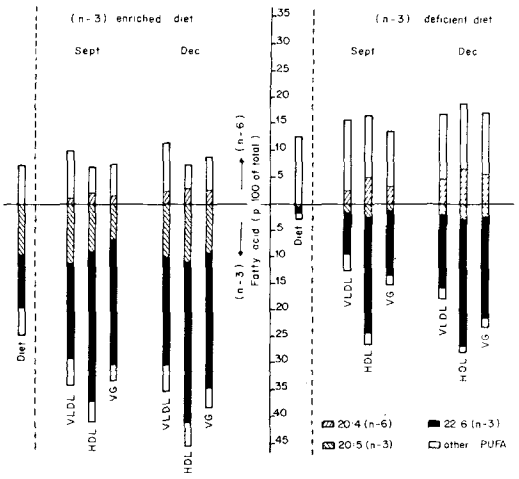

FIG. 3. Distribution of dietary (n-3) and (n-6) PUFA, serum VLDL, HDL and vitellogenin at the third month of vitellogenesis (September) and at ovulation (December). Data are expressed in percentage of total fatty acids: (n-6) PUFA: top of diagram; (n-3) PUFA bottom of diagram.

The quantitative aspect of essential fatty acid transport estimated by the calculations is shown on Table 2 and Figure 4. PL carried ca. 55% of total (n-3) PUFA in September and 70% in December, whatever diet was ingested. For a given diet, the total serum levels of 20:5, as well as of 22:6, remained in the same range,

TABLE 2

Contents of Total (n-3) PUFA, 20:5 and 22:6 in Esterified Lipids of Trout Serum (mg/dl)

Total (n-3) PUFA / 20:5 \ 22:6	(n-3) Enriched diet				(n-3) Deficient diet			
	September		December		September		December	
	LP (d < 1.21 g/ml)	VG	LP (d < 1.21 g/ml)	VG	LP (d < 1.21 g/ml)	VG	LP (d < 1.21 g/ml)	VG
In esterified lipid								
Cholesterol ester	24 6 16	6 1 4	30 7 19	9 2 6	t[a] t t	t t t	20 3 17	6 t 5
Triglyceride	284 78 178	51 10 26	97 23 62	89 21 59	144 14 114	28 3 23	47 4 40	33 4 27
Phospholipid	360 106 224	140 24 73	306 73 197	208 50 139	181 17 144	25 3 20	191 18 163	92 10 76

[a]t = trace amounts < 1 mg/dl.

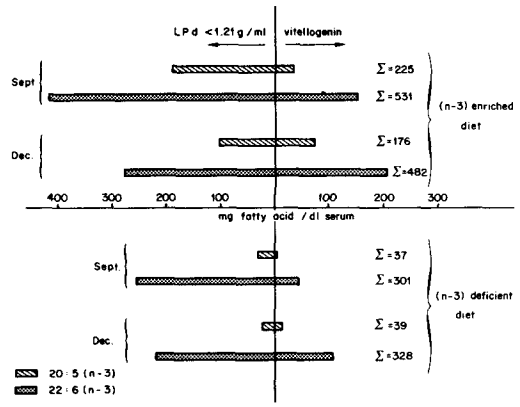

FIG. 4. Contents of total (n-3) PUFA (20:5 and 22:6) in LP (d < 1.21 g/ml) and in vitellogenin of trout serum (mg/dl).

i.e., the higher amount in LP (d < 1.21 g/ml) corresponded to the lower amount in VG.

The overall effect of (n-3) deficiency on serum levels of (n-3) fatty acids was a 55% decrease in September and a 48% decrease in December. 20:5 was affected more drastically; it fell by 6 times and 4.5 times, whereas 22:6 only fell by 1.7 times and 1.5 times.

DISCUSSION

The high level of VLDL during vitellogenesis is consistent with the well-known effect of estrogens in oviparous vertebrates, i.e., the induction of liver biosynthesis of VLDL and VG (13-15). In the present work, the higher serum VG content in December might represent an excess of the complex previously synthesized and subsequently catabolized by another pathway. Serum estrogen levels are usually low at spawning time. In a normal 1-year seasonal cycle, estradiol levels in female trout serum rise at the onset of exogenous vitellogenesis, the maximum being reached in October (16,17) or at the end of vitellogenesis, some weeks before spawning (2). The VG levels found by Whitehead et al. (16) were 1250 mg/dl in September and 2800 mg/dl in December. On the other hand, van Bohemen (18) recovered 100 mg/dl or less during previtellogenesis and endogenous vitellogenesis. These levels varied during exogenous vitellogenesis, reaching 1290 mg/ml in November and decreasing during the following months.

These authors' values are lower than the ones we obtained by chemical analysis of the components, but note in all cases, even with the (n-3)-deficient diet, VG levels were 2 times higher at spawning time than during exogenous

vitellogenesis. Some observations suggest that, in addition to VG, circulating LP (d < 1.21 g/ml) may enter the ovary of trout. In the previous experiment (3), the higher levels of (n-3) PUFA in lipovitellin than in VG suggested that LP (d < 1.21 g/ml), which was the vehicle for more (n-3) PUFA than VG, contributed to oocyte lipids. In addition, LP uptake by ovary, at least of VLDL, has now been largely proved in other oviparous species, such as hen. Using labeled amino acids, Holdsworth et al. (19) demonstrated in 1974 the direct transfer of serum VLDL protein into egg LP. Later, Evans et al. (20) observed that the basal lamina was permeable to circulating VLDL of the laying fowl.

If some data lead us to speculate on VLDL uptake by ovary, very little is known about the fate of the HDL class that reaches very high levels during gonadal development in both sexes. In a previous experiment (8), serum HDL levels in 2 groups of spermiating trout were higher than 1000 mg/dl, and this class accounted for more than 50% of the total LP. On the contrary, in a group where most fish were sexually immature, VLDL and LDL were the predominant classes in September. These findings underline the relationships between plasma LP profiles and sexual maturation, but the regulation of HDL levels and the effects of hormones (sexual and others) are still unknown.

The absence of any effect of (n-3) deficiency on the protein-lipid ratio of VG at a given season suggests that the organization of the complex was not affected. The higher value in December than in September may reflect a protein enrichment from the onset of vitellogenesis to spawning. Regular analysis throughout the annual cycle is required to test eventual changes in the component distribution of VG. The values reported by Wiegand and Idler (21) are in the same range as ours because they found 79% protein and 21% lipid.

In all studies, phospholipid is the predominant lipid class, accounting for more than 70% by weight. The VG composition of other species of fish studied is similar; in the elasmobranch, *Scyliorhinus canicula* (22), and in goldfish (23) lipid represents 18% and 21% of total VG.

The present data clearly show that the effect of the (n-3) deficient diet on plasma fatty acids was more pronounced in September than in December and that VG was more affected than LP (d < 1.21 g/ml). In the previous study (3), the diet deficient in (n-3) was only ingested during the last 3 months of vitellogenesis. When comparing results obtained in December, the trout of the present experiment were subjected to extended depletion. Consequently, they had to mobilize stored (n-3) PUFA throughout the 6 months of exogenous vitellogenesis, and possibly the mechanisms for mobilization and bioconversion became more intense during the last 3 months, when oocyte growth was at its peak. Thus, the process of adapting to the deficient diet during this period might enhance EFA incorporation into VG, which constitutes a reserve of food and structural lipids for the future embryo.

The change in the ratio 22:6/20:5 was caused mainly by an almost complete absence of 20:5, but 20:5 and 22:6 are both essential to growth (24). Recently, the influence of essential fatty acid (EFA) deficiency has been reviewed by Watanabe (25), who found that trout fed an EFA-deficient diet for 3 months before spawning matured but that egg production decreased, the hatching rate was low and some fish larvae were deformed.

Our experiments did not study this question, but chemical analysis showed that a (n-3) deficiency during the last 3 months of vitellogenesis had a moderate effect on 22:6 (n-3) incorporation into egg lipids, whereas 20:5 decreased by 50% (3).

In mammals, PUFA (n-6 and n-3) are esesential to the developing brain and may also play a role in the excitation of nerves (26). This feature suggests that in fish the maternal deficiency of 20:5 and 22:6 may affect embryonic development and larval mobility.

In fish, the oxidative pathways of 20:5 metabolism are not yet widely studied but hydroxy derivatives of 22:6 were recently found in trout gills (27).

Our data lead to the conclusion that a diet deficient in (n-3), given to trout during exogenous vitellogenesis, did not affect the lipoprotein profile but changed PUFA distribution in serum lipids. The very low levels of 20:5 in VG and the fact that 22:6 was incorporated into the complex to a lesser extent during the early stages of oogenesis than at spawning might cause structural oocyte damage and affect the reproductive process.

REFERENCES

1. Campbell, C.M. (1978) Ann. Biol. Anim. Bioch. Biophys. 18, 1013-1018.
2. Lambert, J.G.D., Bosman, G.I.C.G.M., van den Hurk, R., and van Oordt, P.G.W.J. (1978) Ann. Biol. Anim. Bioch. Biophys. 18, 923-927.
3. Léger, C., Frémont, L., Marion, D., Nassour, I., and Desfarges, M.F. (1981) Lipids 16, 593-600.
4. Fulco, A., and Mead, J. (1959) J. Biol. Chem. 234, 1411-1416.
5. Léger, C. (1978) Oceanis 4, 367-393.
6. Frémont, L., Léger, C., and Boudon, M. (1981)

Comp. Biochem. Physiol. 69B, 107-113.
7. Havel, R.J., Eder, H.A., and Bragdon, J.H. (1955) J. Clin. Invest. 34, 1345-1353.
8. Frémont, L., and Marion, D. (1982) Comp. Biochem. Physiol. 73B, 849-855.
9. Folch, J., Lees, M., and Sloane Stanley, G.H. (1957) J. Biol. Chem. 226, 497-509.
10. Bartlett, G.R. (1959) J. Biol. Chem. 234, 466-468.
11. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
12. Flanzy, J., Boudon, M., Léger, C., and Pihet, J. (1976) J. Chromat. Sci. 14, 17-24.
13. Wittliff, J.L., and Kenny, F.J. (1972) Biochim. Biophys. Acta 269, 485-492.
14. Chan, L., Jackson, R.L., O'Malley, B.W., and Means, A.R. (1976) J. Clin. Invest. 58, 368-379.
15. Williams, D.L. (1979) Biochemistry 18, 1056-1063.
16. Whitehead, C., Bromage, N.R., Forster, J.R.M., and Matty, A.J. (1978) Ann. Biol. Anim. Bioch. Biophys. 18, 1035-1043.
17. Whitehead, C., Bromage, N.R., Breton, B. (1983) Aquaculture 34, 317-326.
18. van Bohemen, C., Lambert, J.G.D., and Peute, J. (1981) Gen. Comp. Endocrinol. 44, 94-107.
19. Holdsworth, G., Michell, R.H., and Finean, J.B. (1974) FEBS Letters 39, 275-277.
20. Evans, A.J., Perry, M.M., and Gilbert, A.B. (1979) Biochim. Biophys. Acta 573, 184-195.
21. Wiegand, M.D., and Idler, R. (1982) Can. J. Zool. 60, 2683-2693.
22. Craik, J.C.A. (1978) Comp. Biochem. Physiol. 60B, 9-18.
23. Hori, S.H., Kodama, T., and Tanahashi, K. (1979) Gen. Comp. Endocrin. 37, 306-320.
24. Takeuchi, T., and Watanabe, T. (1976) Bull. Japan. Soc. Scient. Fish 42, 907-919.
25. Watanabe, T. (1982) Comp. Biochem. Physiol. 73B, 3-15.
26. Menon, N.K., and Dhopeshwarkar, G.A. (1982) in Progress in Lipid Research (Holman, R.T., ed.) Vol. 21, pp. 309-326, Pergamon Press Ltd., Oxford, U.K.
27. German, B., Bruckner, G., and Kinsella, J. (1983) Prostaglandins 26, 207-210.

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Effects of Cyclopropene Fatty Acids on the Lipid Composition of the Morris Hepatoma 7288C

RAPHAEL A. ZOELLER and **RANDALL WOOD**, *Department of Biochemistry and Biophysics, Texas Agricultural Experiment Station, Texas A&M University System, College Station, TX 77843*

ABSTRACT

Fatty acids of *Sterculia foetida* were added to the medium used to maintain the Morris hepatoma 7288C in culture. The effect of this supplement on the lipid composition was examined. Overall, monoene levels were decreased with 18:1 levels reduced by 40%. Saturated fatty acid levels were increased, with stearate (18:0) levels 220% of control values. No effect occurred on the level of polyunsaturates (18:2, 20:4, 22:5, 22:6). These changes in fatty acid makeup were observed in both neutral and phospholipid fractions, and all lipid classes were affected. Triglycerides were most affected with a 66% decrease in 18:1. There appeared to be little specificity of effect in the phospholipids with 18:1 levels decreased 40-60% in all classes. All classes were therefore dependent on an endogenous supply of 18:1. Examination of the distribution of geometrical isomers of 18:1 reveals that in all lipid classes, except diphosphatidylglycerol (DPG), the ratio of $\Delta 11$ to $\Delta 9$ isomer decreased toward the isomeric distribution displayed by total medium lipids. In DPG, although 18:1 levels were lowered, the isomeric distribution increased. DPG, synthesized and found in the mitochondria, may use a separate pool of 18:1 during synthesis. Cyclopropene fatty acids (sterculic and malvalic) were incorporated into both neutral and phospholipid fractions with preferential incorporation into triglycerides. Cyclopropene fatty acids were not selectively incorporated into any phospholipid species. Sphingomyelin did not incorporate cyclopropene fatty acids, indicating that a different class of acyltransferase is used in the formation of this phospholipid class.
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INTRODUCTION

The Morris hepatoma 7288C (HTC), grown in tissue culture, exhibits abnormally high levels of 18:1 when compared with liver. Particularly noticeable are the high levels of 18:1 in the phosphoglycerides, major components of the membranes. This aberration in fatty acid composition is not unique to this cell line, but rather is a common phenomenon, observed among a variety of hepatoma cell lines (1-5). The fatty acid composition of the HTC cell is carefully maintained. Alterations in the serum content of medium used to maintain the Morris hepatoma cells in culture and growth in fat-free medium fail to effect changes in the lipid composition of these cells (5). The ubiquity of elevated 18:1 levels and the ability of this cell line to maintain a particular fatty acid composition indicate that maintenance of 18:1 levels in, at least the phospholipid fraction of neoplastic cells, is of importance in maintenance of proper cellular function.

The source of 18:1 in these cells is presently not well defined. The growing cells may obtain 18:1 either through incorporation of 18:1 from the surrounding medium (exogenous) or through synthesis within the cell (endogenous). Synthesis within the cell may be accomplished, through the stearoyl-CoA desaturase system, either by production, from acetyl-CoA and malonyl-CoA, through de novo synthesis, or through the desaturation of exogenous stearate (18:0). The contribution that these pathways make toward the 18:1 requirements of this cell line during rapid growth is unknown. Also in question is the dependence of individual neutral and phospholipid species on endogenously or exogenously supplied 18:1. Considering the divergent pathways involved, certain classes may rely more heavily on different fatty acid pools. Rapidly growing cells may selectively maintain high levels of 18:1 in certain lipid classes, particularly the membrane phospholipids, because of their importance in cellular function. Alterations in membrane fatty acid composition have been shown to affect the activity of membrane-bound enzyme (6), including the mammalian stearoyl-CoA desaturase system (7), as well as the permeability of membranes to nonionic small molecules, e.g., glycerol, ethanol and so forth (8). The ability of normal liver to selectively maintain the fatty acyl integrity of phospholipids has been demonstrated (9).

Sterculic acid, an inhibitor of the stearoyl-CoA desaturase system (10,11), was added to the medium used to grow the Morris hepatoma 7288C in order to determine the importance of this desaturase system for the maintenance of high 18:1 levels. The individual neutral and

phospholipid classes were examined to determine the ability of neoplastic cells to preserve the fatty acid composition of specific lipid classes.

MATERIALS AND METHODS

Preparation of *Sterculia foetida* Fatty Acids

Sterculia foetida seeds were purchased from United Chemical and AP, Calcutta, India. Its seed oil was obtained by extracting the crushed seed kernel with hexane. Solvent was removed by flash evaporation and fatty acids were prepared by saponification with 1N KOH in 70% ethanol for 1 hr at 100 C. Fatty acids were extracted after neutralization with hexane and purified on thin layer chromatography (TLC) using Silica gel G (MC/B Manufacturing Chemists, Inc., Cincinnati, OH) in a chloroform solvent system. Analysis of fatty acid methyl esters on gas liquid chromatography (GLC) revealed that sterculic acid constitutes 57.4% of the fatty acid mass. Other fatty acids were palmitate (13.1%), malvalate (14.5%), octadecenoate (5.6%), linoleate (5.2%), stearate (1.6%) and unknowns (2.6%). *Sterculia foetida* fatty acids were added to the culture medium in ethanol. Final ethanol concentration in the medium was 0.1%.

Growth of HTC Cells

Morris hepatoma 7288C cells were plated in roller culture flasks and allowed to attach overnight in Swim's 77 minimal essential medium supplemented with $NaHCO_3$ (2.2 g l^{-1}), glutamine (292 mg l^{-1}), $CaCl_2 \cdot 2H_2O$ (265 mg l^{-1}), cystine (12 mg l^{-1}), penicillin (60 mg l^{-1}), phenol red (5 mg l^{-1}), fetal calf serum and calf serum (5% by volume each). This medium is designated "5/5." After attachment, the medium was removed and 100 ml of 5/5 medium containing fatty acids of *S. foetida* (20 μg ml^{-1}) was added. To control cells was added 100 ml of 5/5 medium containing 0.1% ethanol. Media were changed daily. Cells were grown for a period of at least 5 doubling times before harvest.

For harvest, medium was removed and cells washed with phosphate buffered saline (PBS), pH 7.4, and released with trypsin. Trypsin was inhibited after 3 min at 37 C with the addition of 5/5 medium and cells were counted using a Neubauer counting chamber. Cells were pelleted at 70 x *g* for 10 min and resuspended in PBS containing defatted bovine serum albumin (2 mg ml^{-1}), remaining on ice for 5 min. Cells were pelleted again and supernatant removed. The cellular pellet was weighed in a tared centrifuge tube and stored at −80 C.

Lipid Analysis

Frozen cells were lypophilized in tared flasks and weighed to obtain dry weight. The total lipid fraction was extracted twice by the modified method of Bligh and Dyer (12). The extracts were combined and solvent removed by flash evaporation. Lipid was redissolved in chloroform and filtered through a fritted glass filter (fine mesh) in order to remove contaminating protein. Phospholipid and neutral lipids were separated using silicic acid chromatography (13). Total lipid, neutral lipid and phospholipid were quantitated gravimetrically after solvent was removed. Phospholipid and neutral lipid classes were separated using thin layer chromatography (TLC) (12). For analysis of geometrical isomers of octadecenoic acid, diphosphatidylglycerol (DPG) was further purified (to remove phosphatidic acid) by TLC using a .5 mm HR plate in chloroform/methanol/30% ammonia (65:25:5, v/v/v). Free fatty acids from the various classes were produced by saponification using 1N KOH in 70% ethanol for 2 hr at 100 C, and extracted 3 times using hexane after the saponification mixture was neutralized with HCl. When quantification was desired, a known amount of internal standard, heneicosanoic acid (21:0), was added before saponification. Methyl esters were produced using diazomethane (14). Fatty acid methyl esters (FAME) were treated with methanol saturated with $AgNO_3$ to produce the methoxy derivatives of sterculic and malvalic acids (15). FAME were analyzed using GLC on a 10% SP2330 column (5 ft) (Supelco, Inc., Bellefonte, PA), temperature programmed from 140 C to 210 C at 3 C/min using a Varian Model 3700 chromatograph. Isomers of 18:1 were separated using a 25 meter OV-101 capillary column at 150 C. Quantitation of the individual phospholipid classes separated by TLC was determined by phosphorus analysis, according to the procedure of Rouser et al. (16). All solvents used in analyses and extractions were distilled in glass (Burdick and Jackson Laboratories, Inc., Muskegon, MI).

RESULTS

Table 1 shows the effects of growth of HTC cells in sterculic acid supplemented medium on total lipid, neutral lipid and phospholipid concentrations. A slight but significant increase ($P < .005$) occurred in dry weight per cell, accompanied by a similar increase ($.01 > P > .005$) in total lipid concentration in the cells supplemented with sterculic acid. This increase was caused primarily by the increased levels of neutral lipids, whereas phospholipid content

TABLE 1

Effect of Cyclopropene Fatty Acids on the Total Lipid, Neutral Lipid and Phospholipid Content of Morris Hepatoma 7288C

	Dry weight ($mg/10^9$ cells)	Total lipid ($mg/10^9$ cells)	Neutral lipid ($mg/10^9$ cells)	Phospholipid ($mg/10^9$ cells)
- CPFA	373 ± 13	50.3 ± 3.5	16.1 ± 1.7	36.1 ± 1.1
+ CPFA	427 ± 12	60.9 ± 2.3	20.2 ± 2.1	37.8 ± 0.9

CPFA = cyclopropene fatty acids.

TABLE 2

Effect of Cyclopropene Fatty Acids on the Phospholipid Profile of the Morris Hepatoma 7288C

	Relative percentage of phospholipid class						
	O	SPH	PC	PI	PS	PE	DPG + PA
- CPFA	1.0 ± 0.5	9.5 ± 0.8	39.2 ± 1.6	9.4 ± 0.4	6.7 ± 0.4	27.2 ± 1.4	5.2 ± 0.1
+ CPFA	1.1 ± 0.3	9.0 ± 0.3	39.4 ± 1.4	9.3 ± 0.3	6.9 ± 0.3	27.9 ± 1.2	5.1 ± 0.1

O = origin of TLC plate, SPH = sphingomyelin, PC = phosphatidylcholine, PI = phosphatidylinositol, PS = phosphatidylserine, PE = phosphatidylethanolamine, DPG = diphosphatidylglycerol, PA = phosphatidic acid, CPFA = cyclopropene fatty acids.

remained unchanged. The increase in neutral lipid content was probably caused by increased triglyceride levels resulting from the sequestering of added fatty acids. Phospholipid levels were unaffected, as was the distribution of phospholipid species (Table 2).

Examination of the effect of sterculic acid on levels of other fatty acids in the total lipids of these cells (Table 3) reveals that 18:1 levels were lowered dramatically (40%). Hexadecenoate (16:1) levels were reduced as well. This reduction in monoenoic fatty acid levels was accompanied by increases in the level of saturated fatty acids 16:0 and 18:0. The polyenoic fatty acid levels (18:2, 20:4, 22:5, 22:6) remained unaffected. Both sterculic acid and malvalic acid, present in the medium, were incorporated into cellular lipids, making up 14.6% and 3.3% of the total fatty acid mass. These effects were observed in both neutral lipid and phospholipid fractions. Table 4 shows that the phospholipid fatty acid profile exhibited the same perturbations observed in the total lipid fraction, with decreased 16:1 and 18:1 and increased levels of the saturates. Neutral lipids were affected to a greater degree because 18:0 was increased 4-5 fold. Both neutral lipid and phospholipid fractions incorporated the cyclopropene fatty acids sterculic and malvalic acids. These were found as 11% of the phospholipid fatty acids while comprising 26% of the total fatty acid mass in neutral lipids.

Examination of the fatty acid composition of the individual neutral lipid classes (Table 5) shows that all 3 classes—triglycerides (TG), free fatty acid (FFA) and sterol esters (SE)—exhibited the same trends as observed before. TG showed the greatest changes, with 18:1 decreasing from 66.2% to 22.2% when cells were treated, and 18:0 increasing from 7.4% to 26.4% (a 3.6-fold increase over the untreated cells). Least affected within the neutral lipids were sterol esters, which showed a decrease from 37.7% to 24.1% in 18:1 and only a 1.9-fold increase in 18:0 levels. Sterculic and malvalic acids were incorporated into all 3 neutral lipid classes, with TG showing the greatest degree of incorporation. Cyclopropene fatty acid made up 26% of the fatty acid mass in this lipid class. Cyclopropenes comprised 16% of the fatty acid mass in the sterol esters whereas FFA showed the least degree of incorporation with cyclopropene fatty acid comprising less than 6% of the total fatty acid mass.

Examination of individual phospholipid classes (Tables 6 and 7) shows that, throughout all classes, the same general trends were observed. Monoenes decreased whereas saturated fatty acids increased. Little selectivity appears for these effects among the phospholipid classes. Sphingomyelin (SPH), which normally contains primarily saturated fatty acids, showed the least amount of change. All of the phospholipid classes incorporated less cyclopropene fatty acids than the neutral lipids. Phospha-

TABLE 3

Effects of Cyclopropene Fatty Acids on Fatty Acid Levels of the Total Lipids in the Morris Hepatoma 7288C

	μg Fatty acid/10^7 cells										
	16:0	16:1	18:0	18:1	18:2	20:1	20:4	22:5	22:6	SA	MA
- CPFA	44 ± 4	12 ± 1	40 ± 4	151 ± 11	21 ± 1	8 ± 1	10 ± 2	4 ± 1	2 ± 1	—	—
+ CPFA	61 ± 5	7 ± 1	100 ± 13	95 ± 4	27 ± 4	10 ± 1	11 ± 2	4 ± 1	2 ± 1	58 ± 8	13 ± 4

CPFA = cyclopropene fatty acids, SA = sterculic acid, MA = malvalic acid.

TABLE 4

Effects of Cyclopropene Fatty Acids on the Fatty Acid Composition of Neutral and Phospholipid Fractions in the Morris Hepatoma 7288C

	Fatty acid percentages										
	16:0	16:1	18:0	18:1	18:2	20:1	20:4	20:5	22:6	SA	MA
Phospholipids											
- CPFA	15.4 ± 0.05	4.0 ± 0.2	17.0 ± 1.1	48.3 ± 0.6	8.0 ± 0.8	1.8 ± 0.2	4.1 ± 0.4	0.9 ± 0.2	0.6 ± 0.2	—	—
+ CPFA	16.5 ± 0.8	1.5 ± 0.2	28.9 ± 1.5	24.3 ± 0.2	10.7 ± 0.4	0.3 ± 0.4	4.6 ± 0.2	1.0 ± 0.2	0.6 ± 0.2	8.9 ± 0.9	1.7 ± 0.1
Neutral lipids											
- CPFA	10.6 ± 0.3	5.2 ± 0.3	7.8 ± 0.2	65.4 ± 0.2	5.2 ± 0.4	5.6 ± 0.1	—	—	—	—	—
+ CPFA	14.5 ± 0.6	1.7 ± 0.2	26.0 ± 1.9	23.6 ± 1.1	4.5 ± 0.1	3.7 ± 0.3	—	—	—	22.2 ± 2.3	3.7 ± 0.7

SA = sterculic acid, MA = malvalic acid, CPFA =cyclopropene fatty acids.

TABLE 5

Effects of Cyclopropene Fatty Acids on the Fatty Acid Composition of Neutral Lipid Classes in the Morris Hepatoma 7288C

Neutral lipid class	Fatty acid percentages							
	14:0	16:0	16:1	17:0	17:1	18:0	18:1	18:2
TG[a]								
-CPFA	1.4 (0.1)[b]	10.2 (0.3)	4.7 (0.5)	0.3 (0.1)	0.5 (0.1)	7.4 (0.5)	66.2 (1.3)	3.4 (1.0)
+CPFA	2.0 (0.1)	14.6 (0.8)	1.1 (0.5)	0.6 (0.0)	0.2 (0.0)	26.4 (2.2)	22.2 (1.4)	3.0 (0.4)
FFA								
-CPFA	2.1 (0.3)	18.6 (1.5)	6.4 (0.9)	0.9 (0.2)	0.5 (0.1)	17.7 (1.9)	46.3 (4.1)	2.9 (0.2)
+CPFA	2.3 (0.3)	23.5 (0.7)	3.0 (1.2)	1.3 (0.1)	0.5 (0.2)	37.9 (1.8)	19.2 (2.5)	3.0 (1.0)
SE								
-CPFA	2.4 (0.5)	11.6 (0.5)	8.7 (0.2)	1.3 (0.1)	1.8 (0.2)	6.6 (0.9)	37.7 (0.6)	18.9 (1.9)
+CPFA	2.8 (0.7)	12.9 (0.9)	6.8 (0.4)	0.9 (0.1)	1.2 (0.2)	12.5 (2.2)	24.1 (0.7)	16.8 (1.3)

Neutral lipid class	Fatty acid percentages						
	18:3	20:1	20:4	24:0	24:1	SA	MA
TG[a]							
-CPFA	–	5.9 (0.7)	–	–	–	–	–
+CPFA	–	4.0 (0.3)	–	–	–	20.3 (4.1)	5.7 (1.7)
FFA							
-CPFA	–	3.8 (0.2)	–	0.8 (0.3)	–	–	–
+CPFA	–	2.2 (1.1)	–	1.4 (0.4)	–	3.7 (0.9)	2.1 (2.4)
SE							
-CPFA	2.8 (1.0)	3.7 (1.0)	1.8 (0.4)	–	1.0 (0.5)	–	–
+CPFA	2.8 (0.3)	2.1 (0.3)	2.6 (1.1)	–	–	12.4 (1.2)	3.6 (1.3)

[a]TG = triglycerides, FFA = free fatty acids, SE = sterol esters, SA = sterculic acid, MA = malvalic acid, CPFA = cyclopropene fatty acid.
[b]Values in parentheses represent the standard deviations from 4 samples.

TABLE 6

Effect of Cyclopropene Fatty Acids on the Fatty Acid Composition of Phosphatidylcholine, Phosphatidylethanolamine and Sphingomyelin in the Morris Hepatoma 7288C

	Fatty acid percentages								
	14:0	16:0	16:1	17:0	17:1	18:0	18:1	18:2	20:0
PC[a]									
-CPFA	0.4 (0.1)[b]	28.1 (1.1)	5.4 (0.3)	0.8 (0.1)	0.7 (0.1)	8.7 (0.5)	45.5 (2.1)	7.5 (0.5)	–
+CPFA	0.6 (0.1)	32.2 (1.4)	1.0 (0.9)	1.1 (0.2)	0.4 (0.1)	20.5 (0.2)	24.7 (0.4)	10.7 (0.4)	–
PE									
-CPFA	–	5.6 (0.6)	2.2 (0.4)	0.4 (0.1)	0.5 (0.1)	16.9 (1.1)	57.9 (1.7)	7.0 (0.3)	–
+CPFA	–	6.8 (0.5)	0.9 (0.3)	0.7 (0.1)	0.4 (0.1)	33.6 (1.7)	27.0 (1.5)	10.4 (1.2)	–
SPH									
-CPFA	1.5 (0.7)	34.6 (1.8)	3.1 (0.3)	1.7 (0.3)	–	19.2 (2.5)	18.1 (3.7)	1.3 (0.6)	0.6 (0.3)
+CPFA	0.8 (0.2)	35.3 (2.0)	1.9 (0.4)	2.4 (0.4)	–	24.4 (3.0)	7.0 (2.5)	1.1 (0.3)	0.8 (0.2)

	Fatty acid percentages								
	20:1	20:4	22:0	24:0	24:1	22:5	22:6	SA	MA
PC[a]									
-CPFA	2.0 (0.3)	0.9 (0.2)	–	–	–	T	T	–	–
+CPFA	1.6 (0.2)	1.1 (0.2)	–	–	–	T	T	4.6 (0.6)	1.5 (1.0)
PE									
-CPFA	1.8 (0.2)	5.9 (0.7)	–	–	–	1.0 (0.2)	0.8 (0.2)	–	–
+CPFA	0.7 (0.2)	7.7 (1.2)	–	–	–	1.7 (0.2)	1.4 (0.2)	5.6 (0.7)	3.1 (0.4)
SPH									
-CPFA	–	–	2.9 (0.9)	7.5 (2.1)	9.5 (2.4)	–	–	–	–
+CPFA	–	–	6.8 (1.0)	14.4 (3.0)	5.1 (1.7)	–	–	–	–

[a]PC = phosphatidylcholine, PE = phosphatidylethanolamine, SPH = sphingomyelin, SA = sterculic acid, MA = malvalic acid, CPFA = cyclopropene fatty acids.

[b]Values in parentheses represent the standard deviations from 4 samples.

TABLE 7

Effects of Cyclopropene Fatty Acids on the Fatty Acid Composition of Phosphatidylinositol, Phosphatidylserine, Diphosphatidylglycerol and Phosphatidic Acid in the Morris Hepatoma 7288C

	Fatty acid percentages						
	14:0	16:0	16:1	17:0	17:1	18:0	18:1
PI[a]							
-CPFA	–	3.5 (0.5)[b]	2.0 (0.3)	0.3 (0.1)	0.3 (0.1)	46.4 (0.7)	39.1 (1.1)
+CPFA	–	3.6 (0.4)	0.4 (0.1)	0.4 (0.1)	0.2 (0.0)	55.6 (0.8)	16.3 (0.6)
PS							
-CPFA	–	4.7 (0.5)	1.7(0.3)	0.6 (0.1)	0.5 (0.1)	34.3 (1.4)	49.3 (0.7)
+CPFA	–	4.9 (0.3)	0.7 (0.2)	0.6 (0.1)	0.3 (0.1)	48.4 (1.4)	24.0 (0.7)
PA + DPG							
-CPFA	1.2 (0.2)	10.0 (0.5)	7.2 (0.7)	0.5 (0.1)	0.9 (0.2)	7.9 (1.1)	48.6 (0.6)
+CPFA	1.4 (0.1)	12.2 (0.8)	2.8 (0.2)	0.7 (0.1)	0.6 (0.1)	15.5 (1.6)	30.8 (0.8)

	Fatty acid percentages						
	18:2	20:1	20:4	22:5	22:6	SA	MA
PI[a]							
-CPFA	2.8 (0.1)	1.6 (0.7)	3.0 (0.5)	1.0 (0.1)	T	–	–
+CPFA	2.8 (0.1)	1.6 (0.1)	2.5 (1.2)	1.3 (0.1)	0.7 (0.1)	8.5 (0.4)	6.1 (0.8)
PS							
-CPFA	4.0 (0.5)	1.8 (0.4)	1.3 (0.4)	1.2 (0.1)	0.6 (0.2)	–	–
+CPFA	6.2 (0.5)	1.1 (0.1)	2.1 (0.7)	2.3 (0.6)	1.6 (0.5)	5.8 (0.7)	2.0 (1.0)
PA + DPG							
-CPFA	19.3 (1.2)	1.0 (0.3)	0.9 (0.2)	1.3 (0.3)	1.2 (0.3)	–	–
+CPFA	26.0 (1.9)	0.5 (0.1)	2.0 (1.1)	2.3 (0.2)	0.6 (0.1)	3.6 (0.6)	1.4 (0.4)

[a]PI = phosphatidylinositol, PS = phosphatidylserine, DPG = diphosphatidylglycerol, PA = phosphatidic acid, SA = sterculic acid, and MA = malvalic acid, CPFA = cyclopropene fatty acids.
[b]Values in parentheses represent the standard deviations from 4 separate samples.

tidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and combined diphosphatidylglycerol and phosphatidic acid all showed cyclopropenes to make up less than 9% of the total fatty acid mass, whereas phosphatidylinositol (PI) incorporated more (14.6%). SPH incorporated no cyclopropenes.

Table 8 shows that, when treated for long periods of time (5 doubling periods), the isomeric profile of octadecenoates tended to simulate that of the medium. Vaccenate constituted 31.5 ± 2.2% of the 18:1 found in the total lipid fraction in control cells, with oleate (Δ9) making up the remainder. Vaccenate constituted only 19.9 ± 1.9% of the total 18:1 content in the treated cells; much closer to the 14.7% value observed in the medium lipids. Examination of the individual lipid classes reveals that all classes, with the exception of DPG, were affected. Most noticeably affected were the phospholipids. PE and PS in treated cells demonstrated a lower vaccenate percentage than that of total medium lipid, and resembled the FFA fraction of the medium. DPG did not show a decrease in vaccenate; in fact the vaccenate percentage increased from 40.5% to 54.5%.

DISCUSSION

Addition of *S. foetida* seed oil to the feed of host animals bearing Morris hepatoma 7288-CTC (9) or to the culture medium used to maintain the Morris hepatoma 7288C (17,18) in previous studies has had little or no effect on the fatty acid profile of this tumor tissue. The addition of FFA of *S. foetida* in this study, however, effected rather drastic changes in the fatty composition of the Morris hepatoma 7288C. The reason for this discrepancy is probably the inability of these cells to incorporate and use fatty acids when esterified, in the form of complex lipids such as TG, as in *S. foetida* seed oil. Of the various forms in which fatty acid may exist in the medium, the FFA form is probably the most readily taken up by cells in culture. FFA, bound to albumin, serve as an excellent substrate for uptake in both neo-

TABLE 8

Effects of Cyclopropene Fatty Acids on Octadecenoate Isomer Percentages in Various Lipid Classes in the Morris Hepatoma 7288C

Lipid class	Percentage of Δ11 isomer[a]	
	-CPFA	+CPFA
Cellular lipids		
TG[c]	35.3 ± 1.1[b]	24.9 ± 0.4
SE	29.9 ± 2.0	23.0 ± 1.2
FFA	41.8 ± 2.8	27.4 ± 1.6
PC	33.4 ± 0.7	18.4 ± 1.4
PE	21.4 ± 1.1	11.9 ± 1.1
SPH	31.4 ± 2.0	17.7 ± 0.8
PI	24.1 ± 1.0	16.0 ± 1.0
PS	16.2 ± 0.1	10.2 ± 1.4
DPG	40.5 ± 3.2	54.5 ± 0.9
Total lipids	31.5 ± 2.2	19.9 ± 1.9
Medium lipids		
FFA	9.4	
TG	20.0	
SE	15.3	
PL	13.5	
Total lipids	14.7	

[a]The Δ9 isomer constituted the remainder of the isomeric makeup in cellular lipid. A small percentage of Δ12 was observed in the serum lipid (>3%). This isomer was not found in cellular lipid and was disregarded in the calculations.

[b]All values for cellular lipid represent the average ± SD for 4 samples. Values for serum lipids represent a single determination.

[c]TG = triglycerides, SE = sterol esters, FFA = free fatty acids, PC = phosphatidylcholine, PE = phosphatidylethanolamine, PI = phosphatidylinositol, PS = phosphatidylserine, SPH = sphingomyelin, DPG = diphosphatidylglycerol, PL = total phospholipids.

plastic (19,20) and non-neoplastic cell lines (21). Complex lipids, e.g., TG, are incorporated into cultured cells, although at much lower rates. Bailey et al. (22), working with L-strain mouse fibroblasts, reported that TG were incorporated at one-tenth the rate of the FFA whereas phospholipid was not used by the cells. *Ehrlich ascites* cells are able to use TG present in the ascites plasma very low density lipoproteins (23,24), although at a much slower rate when compared to FFA. Sterculic acid, when presented as the oil (primarily as triglycerides) in the culture medium, can reasonably be assumed not to be incorporated into the HTC cells and therefore had no effect. When fed to the host animal bearing the Morris hepatoma 7288CTC (9), sterculic acid may have been sequestered by the liver and never actually presented to the hepatoma, or, if it is present in the surrounding fluid, it is again esterified in complex lipids and not readily incorporated. Hyperalimentation or intravenous injection of sterculic acid may yield alterations in the fatty acid composition of solid tumors maintained in the host.

The addition of cyclopropene fatty acids caused a 40% reduction of 18:1 levels in these cells. Sterculic acid inhibits the formation of monoenoic fatty acids (11), and recently, results from this lab have shown that sterculic acid is able to inhibit monoene synthesis through de novo synthesis and by desaturation of exogenous stearate in this cell line. The decrease in monoene levels may be caused by the inhibition of the stearoyl-CoA desaturase system, and thus the synthesis of 18:1 within the cell, and the inability of exogenous 18:1 to become incorporated because of competition from the added *S. foetida* fatty acids. Although the latter may be involved, the major factor involved in the reduction of 18:1 levels is probably the inhibition of the stearoyl-CoA desaturase system. The levels of other fatty acids, including linoleic acid (an essential fatty acid), are not reduced. In fact, saturated fatty acid levels are increased. This increase is caused by synthesis or incorporation of 18:0, which would normally be desaturated to form 18:1. The data suggest that the stearoyl-CoA desaturase system, although low in this tissue when measured in vitro (25) in these cells, is responsible for a major portion of the 18:1 observed. This may be the case in other hepatoma cell lines (26-28), which display low levels of desaturase activity.

Cyclopropene fatty acids (sterculic acid) effected alterations in the fatty acid profile of both neutral lipids and phospholipids. All lipid classes were dependent on the stearoyl-CoA desaturase system because all neutral and phospholipid classes were affected. These results contrast with those obtained from normal rat liver (9). Although fed as the oil, cyclopropenes were able to affect the lipid composition of rat liver. The liver of rats given a diet supplemented with 0.5% *S. foetida* seed oil demonstrated decreased levels of 18:1 in the neutral lipids whereas phospholipid fatty acid composition was unaffected. Although 18:1 levels were unaffected in phospholipids, the vaccenate (Δ11) to oleate (Δ9) ratio decreased in this fraction, indicating an alteration in the source of 18:1 for phosphoglycerides. The maintenance of phosphoglyceride 18:1 levels may have been achieved through the selectivity of acyltransferases for monoenoic fatty acids during phosphoglyceride synthesis or through a deacylation-reacylation process (Land's cycle) (29) once synthesis is complete. An alternative route of oleate biosynthesis, insensitive to sterculic acid, has been suggested as a possible source of 18:1 in liver (9). Alterations in acyltransferase

activities (30,31), as well as activities of the Land's cycle (31), have been demonstrated in neoplastic tissue. Disfunction of these regulatory mechanisms in the hepatoma may explain this tissue's inability to maintain phosphoglyceride fatty acid composition.

Sterculic and malvalic acids were incorporated into most lipid classes of HTC cells with preferential incorporation into triglycerides. Incorporation of sterculic acid into the complex lipids of normal tissue (trout liver) was demonstrated using radioactively labeled sterculic acid (32). Label was incorporated primarily into the triglyceride fraction in this tissue as well. The distribution of cyclopropene fatty acid in normal rat liver has not been examined. SPH was the only lipid class that did not incorporate cyclopropene fatty acids. SPH differs from other phospholipid species in its fatty acid composition, route of synthesis and the nature of the bond with which the fatty acid moiety is attached (amide linkage instead of an ester linkage). A separate class of acyltransferases is probably involved in the attachment of the acyl moiety.

If sterculic acid inhibits monoene synthesis and there is no specificity for fatty acid uptake of oleate vs vaccenate from the medium, or of these isomers into complex lipids, the ratio of oleate to vaccenate should mimic that of the medium in cells treated with this inhibitor. This indeed is observed. Almost every lipid class, both phospholipid and neutral lipid, demonstrated a decrease in vaccenate relative to oleate. The cells do not appear to distinguish between these 2 isomers.

Analysis of the FFA fraction of sera used to supplement this medium shows that the $\Delta 11$ isomer constitutes only 9% of the 18:1. The shift in vaccenate percentages in most classes of lipid, but most noticeably PE and PS, are consistent with the hypothesis that these cells preferentially use the FFA fraction of the serum-supplemented medium as the preferred source of fatty acid.

The one lipid class that appears to be unaffected by inhibition of the desaturase system is cardiolipin (DPG). The $\Delta 11$ isomer percentage increased in this class. This phospholipid is found primarily in the mitochondria (33), which is also the site of its synthesis (34). The fatty acid composition is probably controlled by selective acylation of the glycerol moiety. Mitochondria possess their own glycerophosphate acyltransferase system (35), which demonstrates distinct positional and fatty acid specificities (36) different from those found in the endoplasmic reticulum. Sterculic acid, however, does lower the 18:1 levels in this lipid class and therefore DPG does depend, to some extent, on the stearoyl-CoA desaturase system.

Through the use of cyclopropene fatty acids we have been able to lower the cellular 18:1 levels 40%. This effect is observed in all lipid classes including the phospholipids, integral components of the membrane systems. This decrease, however, had little effect on any other observable parameter of cellular function, including phospholipid composition (Table 3) and growth rate. The levels of 18:1 required for rapid growth of these cells is unknown. Attempts to further lower 18:1 levels and to evaluate the data obtained in this system are complicated by the presence of exogenous lipids (in a variety of forms) in the serum used in the culture medium. Efforts are presently underway to develop serum-free culture medium for further studies.

REFERENCES

1. Howard, B., Butler, J.D., and Bailey, J.M. (1972) in Tumor Lipids: Biochemistry and Metabolism (Wood, R., ed.) pp. 200-214, American Oil Chemists' Society, Champaign, IL.
2. Van Hoeven, R.P., and Emmelot, P. (1972) in Tumor Lipids: Biochemistry and Metabolism (Wood, R., ed.) pp. 126-138, American Oil Chemists' Society, Champaign, IL.
3. Morton, R., Cunningham, C., Jester, R., Waite, M., Miller, N., and Morris, H.P. (1976) Cancer Res. 36, 3246-3254.
4. Reitz, R.C., Thompson, J.A., and Morris, H.P. (1977) Cancer Res. 37, 561-567.
5. Wood, R.,and Falch, J. (1973) Lipids 8, 702-710.
6. Mazliak, P. (1977) in Lipids and Lipid Polymers in Higher Plants (Trevini, M., and Lichtenthaler, H.K., eds.) pp. 48-74, Springer-Verlag, NY.
7. Enoch, H.G., Catalá, A., and Strittmatter, P. (1976) J. Biol. Chem. 251, 5095-5103.
8. Van Deenan, L.L.M. (1965) in Progress in the Chemistry of Fats and Other Lipids (Holman, R.T., ed.) Vol. 8, pp. 1-27, Pergamon Press Inc., Oxford, England.
9. Wood, R., Chumbler, F., and Weigand, R.D. (1978) Lipids 13, 232-238.
10. Raju, P.K., and Reiser, R. (1972) J. Biol. Chem. 247, 3700-3701.
11. Jeffcoat, R. (1977) Lipids 12, 480-485.
12. Wood, R. (1973) Lipids 8, 690-701.
13. Bergstrom, B. (1952) Acta Physiol. Scand. 25, 101-110.
14. Schlenk, H., and Gellerman, J.L. (1960) Anal. Chem. 32, 1412-1414.
15. Schneider, E.L., Loke, S.P., and Hopkins, D.T. (1968) J. Am. Oil. Chem. Soc. 45, 585-590.
16. Rouser, G., Siakotos, A.M., and Fleischer, S. (1966) Lipids 1, 85-86.
17. Wood, R., and Falch, J. (1974) Lipids 9, 979-986.
18. Wood, R., Falch, J., and Weigand, R.D. (1974) Lipids 9, 987-992.
19. Fillerup, D.L., Migliore, J.C., and Mead, J.F. (1958) J. Biol. Chem. 233, 98-101.
20. Spector, A.A., and Steinburg, P. (1967) Cancer Res. 27, 1587-1594.

21. Howard, B.V., and Kritchevsky, D. (1969) Biochim. Biophys. Acta 187, 293-301.
22. Bailey, J.M., Howard, B.V., and Tillman, S.F. (1973) J. Biol. Chem. 248, 1240-1247.
23. Brenneman, D.E., and Spector, A.A. (1973) Fed. Proc. 32, 2587.
24. Wright, J.D., and Green, C. (1971) Biochem. J. 123, 837-844.
25. Zoeller, R.A., and Wood, R. (1982) J. Am. Oil Chem. Soc. 59, 267.
26. Morton, R.E., Hartz, J.W., Reitz, R.C., Waite, B.M., and Morris, H.P. (1979) Biochim. Biophys. Acta 573, 321-331.
27. Prasad, M.R., and Joshi, V.C. (1979) Lipids 14, 413-415.
28. Raju, P.K. (1974) Lipids 9, 795-797.
29. Hill, E.E., and Lands, W.E.M. (1970) in Lipid Metabolism (Wakil, S.J., ed.) pp. 185-277, Academic Press, Inc., NY.
30. Stern, W., and Pullman, M.E. (1978) J. Biol. Chem. 253, 8047-8055.
31. Waite, M., Parce, B., Morton, R., Cunningham, C., and Morris, H.P. (1977) Cancer Res. 37, 2902-2908.
32. Eisele, T.A., Parker, R.S., Yoss, J.K., Nixon, J.E., Pawlowski, N.E., and Sinnhuber, R.O. (1979) Lipids 14, 523-528.
33. Pugh, E.L., and Kates, M. (1979) Lipids 14, 159-165.
34. Prebble, J.N. (1981) Mitochondria, Chloroplasts and Bacterial Membranes, pp. 122-124, Longman, Inc., NY.
35. Van der Bosch, H. (1974) Ann. Rev. Biochem. 43, 243-277.
36. Monroy, G., Rola, F.H., and Pullman, M.E. (1972) J. Biol. Chem. 247, 6884-6894.

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A Possible Regulatory Role of Squalene Epoxidase in Chinese Hamster Ovary Cells

HAVIVA EILENBERG and **ISHAIAHU SHECHTER**, *Department of Biochemistry, The George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel*

ABSTRACT

Growth of Chinese Hamster Ovary (CHO) cells in the presence of 20% lipid depleted serum (LDS) for only 2 hr results in an increase in the synthesis of [^{14}C]sterols from [^{14}C]mevalonate and from [^{14}C]squalene compared with cells grown under normal growth conditions in the presence of 10% fetal calf serum (FCS). This enhanced sterol synthesis increases with time of exposure of the cells to LDS. However, exposing these cells for time periods up to 42.5 hr to a growth medium containing 20% LDS did not result in enhanced [^{14}C]sterol synthesis from [^{14}C]2,3-oxidosqualene. Incubation of these cells with [^{14}C]mevalonate resulted in the accumulation of [^{14}C]squalene regardless of the presence of either LDS or FCS. These results suggest that squalene epoxidase is a regulatory enzyme in the cholesterol biosynthetic pathway in CHO.
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INTRODUCTION

The major regulatory enzyme in cholesterol synthesis is considered to be 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) (1). However, recent studies suggest that other enzymes of the cholesterol biosynthetic pathway are also regulated simultaneously in this pathway (2-4). The importance of squalene epoxidase in the control of cholesterogenesis has been suggested by a few groups of investigators (5-7). Gonzalez et al. have suggested a regulatory role for squalene epoxidase in cultured human renal cancer cells (7). In addition, squalene epoxidase activity but not oxidosqualene-lanosterol cyclase activity has been shown to be at low levels in mammalian tissues with limited capacity for sterol synthesis, suggesting that squalene epoxidase limits the rate in these cells (5). In this communication, we present evidence that in Chinese hamster ovary (CHO) cells, squalene epoxidase undergoes regulation under different growth conditions. Our results also suggest that squalene epoxidase might limit the rate of cholesterogenesis in these cells and therefore has an important function in the control of cholesterol metabolism.

MATERIALS AND METHODS

Materials

DL-[2-^{14}C]mevalonate (31 mCi/mmol) was purchased from the Radiochemical Centre, Amersham. [^{14}C]Squalene was biosynthesized in our laboratory from [^{14}C]mevalonate according to the method of Popjak (8) with slight modifications (6). [^{14}C] 2,3-Oxidosqualene was biosynthesized in our laboratory by the same procedure as [^{14}C]squalene except that the plant growth retardant Amo 1618 (2-isopropyl-4-[trimethylammonium chloride]-5-methylphenyl-piperidine-1-carboxylate) was added to inhibit squalene-2,3-oxide lanosterol cyclase (9,10). Fetal-calf serum and F-12 medium were from Maagar, Israel. Lipid-depleted serum (44.5 mg/ml) was prepared according to the method of Cham and Knowles (11). All chemicals were of analytical grade.

Cells

CHO cells were grown in 80 cm^2 tissue culture flasks at 37 C in F-12 medium containing 10% fetal calf serum, 2 mM glutamine, penicillin 100 units/ml and streptomycin 100 μg/ml. The cells were harvested using a trypsin (0.25%) −EDTA (0.05%) solution and plated on 50 mm tissue culture plates in 3 ml growth medium.

Preincubation of Cells for the Assay

When the cells reached confluence (3-5 days), the medium was drained off and the cells were rinsed twice with 3 ml phosphate buffered saline (PBS). Then, each plate was preincubated for various time periods with F-12 medium containing either 20% lipid-depleted serum or 10% fetal-calf serum.

Incorporation of Radiolabeled Substrates

In experiments where incorporation of [^{14}C]mevalonate to sterols was determined, the radiolabeled substrate was added directly to the

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preincubation medium and the incubation was carried on for 22 hr. On the other hand, when [^{14}C]squalene or [^{14}C]2,3-oxidosqualene incorporation to sterols was studied, the different preincubation media were removed and the cells rinsed twice with 3 ml PBS. Each dish was then incubated with 1.5 ml PBS containing 15 mM glucose, 1% BSA, [^{14}C]squalene or [^{14}C]2,3-oxidosqualene and a final amount of 10% dimethyl sulfoxide (v/v). After various time periods, as indicated in the various experiments, the incubation of the cells with radiolabeled substrates were stopped by adding 160 μl solution of 10% Triton X-100. After 15 min at room temperature, the resulting lysate was transferred to glass tubes and the dishes were washed with 1 ml of 1% Triton X-100 solution followed by 2.5 ml solution of 10% methanolic KOH.

Lipid Saponification and Extraction Procedure

The combined extract from each dish was saponified for 30 min at 55 C. Thereafter, 1 ml acetone was added to each tube and the non-saponifiable materials were isolated by 3 extractions of 2 ml hexane.

Lipid Analyses and Determination

Ten μl of 0.5% (w/v) unlabeled squalene, lanosterol and cholesterol were added as carriers to each combined extract, which was then evaporated under nitrogen. The residues were dissolved in small volumes of benzene and ethylacetate and applied to 0.25 mm thin layer chromatography (TLC) silica gel plates. The plates were then developed for 10 cm with 10% ethylacetate in benzene for analyses of products from [^{14}C]squalene or [^{14}C]2,3-oxidosqualene. For analyses of products from [^{14}C]mevalonate, the samples were applied to silica gel plates impregnated with 1.5% silver nitrate and were developed for 10 cm with hexane/ethylacetate/benzene, 380:120:1. The positions of radiolabeled squalene, lanosterol and cholesterol on the TLC plates was identified with authentic standards. The appropriate zones of the gel were scraped off and counted for radioactivity. Exact identification of [^{14}C]-squalene was done by cochromatography of the radiolabeled material with an authentic standard of squalene on silica gel plates developed with n-hexane ($R_f = 0.45$). Lanosterol and cholesterol were also identified by cochromatography with authentic standards on silica gel plates developed with 10% ethylacetate in benzene or on $AgNO_3$ plates developed with hexane/ethylacetate/benzene, 380:120:1. The radiolabeled products were also identified by GLC (HP model 5790). Squalene was separated and identified on a SP-2340 column and sterols were identified on a 3% SE-30 column. Results are expressed as total radiolabeled sterols produced per dish or per mg protein. Protein was determined according to the method of Lowry et al. (12).

RESULTS AND DISCUSSION

Post HMG-CoA reductase regulation of cholesterol biosynthesis was studied in CHO cells by monitoring the incorporation of the radioactively labeled, post HMG-CoA intermediates, mevalonate, squalene and 2,3-oxidosqualene, into lipids under different growth conditions.

We first determined the conditions that were necessary for the incubation of the radiolabeled substrates with the cultured cells. To do that, we pretreated the cells with 20% LDS for 24 hr. Under these conditions, the cells are known to have an increased endogenous sterol synthesis from acetate caused by the lack of uptake of exogenous cholesterol via the LDL receptor (13). We compared production of [^{14}C]cholesterol from [^{14}C]squalene by these cells to that in cells grown under normal conditions (10% FCS). Figure 1 shows the time-dependent conversion of [^{14}C]squalene into [^{14}C]cholesterol by the cells. In both cases, the accumulation of [^{14}C]cholesterol increased nearly linearly with the time of incubation up to incubation periods of 5 hr. In our assays, we chose to use 4 hr incubation of cells with substrates. This assures both linearity of reaction rate and sufficient conversion of substrates to products for accurate determinations. As can also be seen in this figure, production of radiolabeled cholesterol from squalene is significantly higher in cells that were preincubated in lipid-depleted serum compared with untreated cells. In fact, a 3.2-4.2 fold increase in [^{14}C]cholesterol production was observed regardless of the incubation period. This increase in cholesterol synthesis was not caused by the difference in the amount of the serum present in the cultures as similar differences in cholesterol synthesis were observed in cultures that were pretreated for 24 hr with 20% LDS and 20% FCS (data not shown). The enhanced conversion of squalene to cholesterol by cells treated with 20% LDS for 24 hr was presumably caused by a deficiency in endogenous cellular cholesterol caused by the LDS. Therefore, we preincubated the cells for various periods of time with 20% LDS, then we measured conversion of radiolabeled squalene to sterols. Indeed, direct correlation was found between the rate of conversion of [^{14}C]-

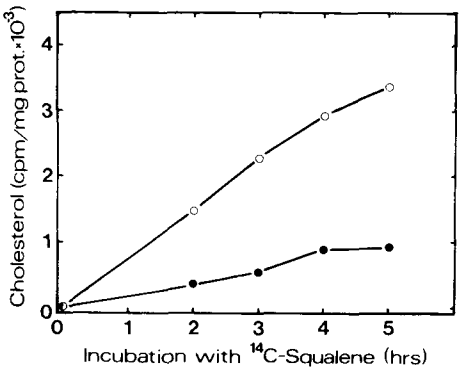

FIG. 1. Time-dependent synthesis of [^{14}C]cholesterol from [^{14}C]squalene by CHO cells. Cells were plated on 50 mm dishes and allowed to reach confluence under normal growth conditions (F-12 supplemented with 2 mM glutamine, penicillin 100 units/ml, streptomycin 100 μg/ml and 10% fetal-calf serum). At confluence, the medium was drained off, the dishes were each rinsed twice with 3 ml PBS and preincubated in 3 ml medium F-12 containing either 10% FCS (●) or 20% LDS (○), for 24 hr. The medium was then removed and the cells rinsed twice with 3 ml PBS. Each dish was incubated with 1.5 ml PBS containing 15 mM glucose, 1% BSA, [^{14}C]squalene (40,000 cpm, 110.7 Ci/mol) and a final amount of 10% dimethyl sulfoxide. The incubation was stopped at various time periods by adding 160 μl solution of Triton X-100. After 15 min at room temperature, the resulting lysate was transferred to glass tubes and the dishes were washed with 1 ml 1% Triton X-100 solution followed by 2.5 ml solution of 10% methanolic KOH. The combined extract was saponified for 30 min at 55 C. The labeled products were then extracted, separated and identified as described in Materials and Methods.

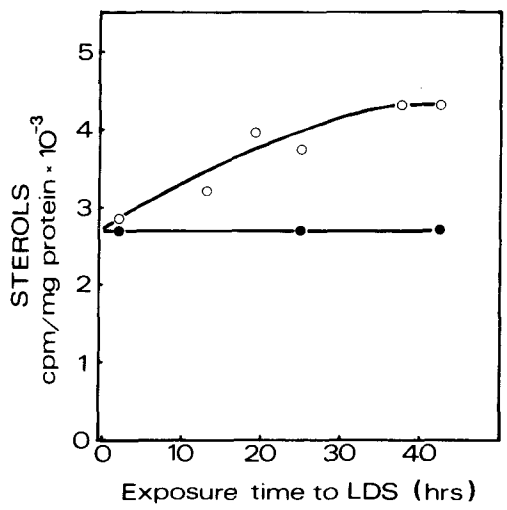

FIG. 2. The effect of incubation of CHO cells with 20% LDS on the incorporation of [^{14}C]squalene into sterols. Cells were plated on 50 mm dishes and allowed to reach confluence under normal growth conditions (F-12 supplemented with 2 mM glutamine, penicillin 100 units/ml, streptomycin 100 μg/ml and 10% fetal-calf serum). At confluence, the medium was drained off and the dishes were each rinsed twice with 3 ml PBS. The dishes were then incubated for various periods of time with 3 ml medium F-12 containing either 10% FCS (●) or 20% LDS (○). At the end of the incubation period, the cells were rinsed twice with 3 ml PBS. Each dish was then incubated with 1.5 ml PBS containing 15 mM glucose, 1% BSA, [^{14}C]-squalene (40,000 cpm, 110.7 Ci/mol) and a final amount of 10% dimethyl sulfoxide. After a 4 hr incubation with the radioactive substrate, the reaction was stopped by addition of 160 μl solution of 10% Triton X-100. After 15 min at room temperature, the resulting lysate was transferred to glass tubes and the dishes were washed with 1 ml 1% Triton X-100 solution followed by 2.5 ml solution of 10% methanolic KOH. The combined extract was saponified for 30 min at 55 C. The labled products were then extracted, separated and identified as described in Materials and Methods.

squalene to [^{14}C]sterols and the time period in which the cells were exposed to LDS (Fig. 2). This rate tends to reach a plateau at ca. 40 hr exposure time to LDS, indicating that a higher steady state level of cholesterol synthesis from squalene was obtained. The new sterol synthesis level obtained is ca. 1.6 higher than that observed in untreated cells, as shown in the same figure. These results not only support previous studies by others that postmevalonate regulation of cholesterol biosynthesis exists, but also suggest the existence of a postsqualene regulation in CHO cells (3,4,7).

Earlier reports suggested that one postsqualene regulatory step in the biosynthesis of sterols might be squalene conversion to 2,3-oxidosqualene by squalene epoxidase (5-7). In order to determine if the increase in the postsqualene sterol synthesis was caused either by an increased squalene epoxidase activity or possibly an increase in some of the subsequent enzymes in the biosynthetic pathway, we compared the rate of sterol synthesis from [^{14}]C]-squalene to that from [^{14}C]2,3-oxidosqualene at various growth conditions. Figure 3 indicates that the rate of sterol synthesis from [^{14}C]-2,3-oxidosqualene did not increase, following exposure of the cells to 20% LDS for various periods of time, compared with cells grown under normal growth conditions. Rather, a decrease in the sterol synthesis from this substrate could be observed in the cells grown in LDS. We do not have, at present, a satisfactory explanation for the observed decrease in sterol synthesis from 2,3-oxidosqualene at short periods of time (up to 40 hr). One possible

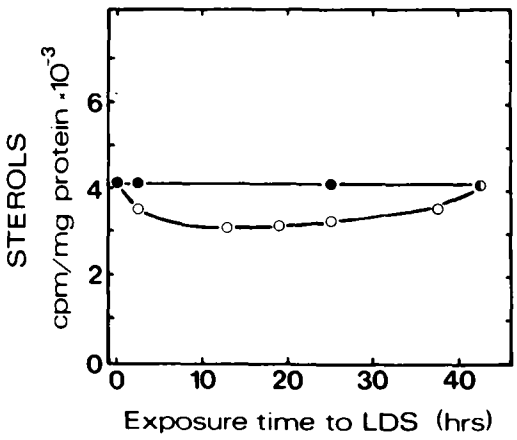

FIG. 3. The effect of incubation of CHO cells with 20% LDS on the incorporation of [^{14}C]2,3-oxidosqualene into sterols. Cells were plated on 50 mm dishes and allowed to reach confluence under normal growth conditions (F-12 supplemented with 2 mM glutamine, penicillin 100 units/ml, streptomycin 100 μg/ml and 10% fetal-calf serum). At confluence, the medium was drained off and the dishes were each rinsed twice with 3 ml PBS. The dishes were then incubated for various periods of time with 3 ml medium F-12 containing either 10% FCS (●) or 20% LDS (○). At the end of the incubation period, the cells were rinsed twice with 3 ml PBS. Each dish was then incubated with 1.5 ml PBS containing 15 mM glucose, 1% BSA, [^{14}C]2,3-oxidosqualene (43,000 cpm, 110.7 Ci/mol) and a final amount of 10% dimethyl sulfoxide. After a 4 hr incubation with the radioactive substrate, the reaction was stopped by addition of 160 μl solution of 10% Triton X-100. After 15 min at room temperature, the resulting lysate was transferred to glass tubes and the dishes were washed with 1 ml 1% Triton X-100 solution followed by 2.5 ml solution of 10% methanolic KOH. The combined extract was saponified for 30 min at 55 C. The labeled products were then extracted, separated and identified as described in Materials and Methods.

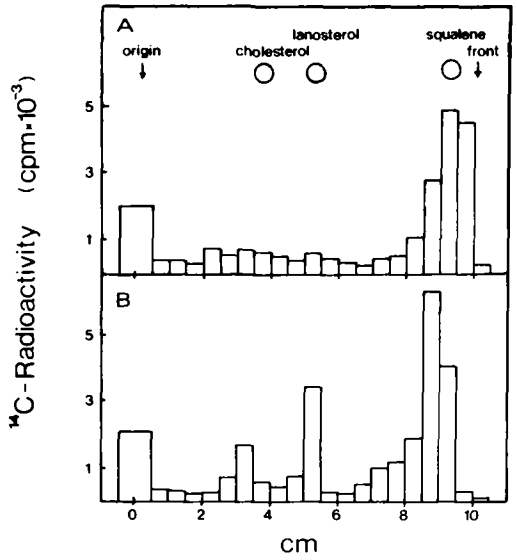

FIG. 4. Incorporation of [^{14}C]mevalonate into nonsaponifiable lipids by CHO cells grown either in 10% FCS or in 20% LDS. Cells were plated on 50 mm dishes and allowed to reach confluence under normal growth conditions (F-12 supplemented with 2 mM glutamine, penicillin 100 units/ml, streptomycin 100 μg/ml and 10% fetal-calf serum). At confluence, the medium was drained off, the plates were each rinsed twice with 3 ml PBS and preincubated with 3 ml F-12 containing either 10% FCS (Panel A) or 20% LDS (Panel B). Twenty-four hours later, 1.25 μCi [^{14}C]mevalonate (26.7 μM) was added to each dish. The incubation with the radioactive substrate was carried on for 22 hr. The incubations were stopped by adding 160 μl solution of 10% Triton X-100. After 15 min at room temperature, the resulting lysate was transferred to glass tubes and the dishes were washed with 1 ml 1% Triton X-100 solution followed by 2.5 ml solution of methanolic KOH. The combined extract was saponified for 30 min at 55 C. The labeled products were then extracted, separated and identified as described in Materials and Methods. The position of authentic standards of cholesterol, lanosterol and squalene are shown on the top. Panel A and B represent the radioactive products obtained from cells grown in the presence of 10% FCS and 20% LDS respectively.

explanation is that the change of the growth media affects various endogenous cell processes, one of which is 2,3-oxidosqualene-lanosterol cyclase; hence, the lowering of this activity. After an initial adjustment period this activity (and possibly other processes as well) increases and, after 45 hr, reaches the level of this activity observed in untreated cells. Whatever the explanation, Figures 2 and 3 clearly show that under conditions that cause enhanced cellular sterol synthesis from squalene (and as will be seen later from mevalonate too), the conversion of 2,3-oxidosqualene to sterols is not enhanced. These findings led us to conclude that a postsqualene regulation may exist in CHO cells at the enzyme squalene epoxidase step. The importance of this enzyme as a regulatory enzyme in the biosynthetic pathway of cholesterol was further confirmed by incorporation studies using [^{14}C]mevalonate as a substrate. Figure 4 illustrates the incorporation of [^{14}]mevalonate into nonsaponifiable lipids in cells grown under normal growth conditions (Panel A) or in medium containing 20% LDS (Panel B). As can be seen in panel A, CHO cells grown in 10% FCS accumulated [^{14}C]squalene whereas sterol synthesis remained low and

barely detectable under the assay conditions. The low rate in sterol synthesis is expected because in the presence of LDL (FCS) this process is suppressed (13). As expected, the rate of sterol synthesis was significantly enhanced when the cells were grown in 20% LDS (Panel B). Yet, even under conditions in which sterol synthesis is enhanced, squalene accumulation is still observed. These results strongly suggest that squalene epoxidase is indeed catalyzing a rate-limiting step, and that in CHO cells this enzyme continues to limit rates even under conditions where increased sterol synthesis occurs. .

In contrast to squalene, 2,3-oxidosqualene is formed in cell-free extracts in very low concentrations (14), suggesting again that the epoxidase activity is a rate-limiting step in sterol synthesis. Therefore, even a small increase in its activity could result in a significant increase in sterol synthesis. The presence of a pool of squalene under both cholesterogenic and noncholesterogenic conditions in CHO cells indicates that the step catalyzed by squalene epoxidase is a significant regulatory step in cholesterol biosynthesis. Havel et al. have shown that in primary cultures of rat hepatocytes, sterol demethylation and HMG-CoA reductase activity were directly related (15). In our studies, we could not observe the accumulation of lanosterol or other precholesterol sterol intermediates from radiolabeled mevalonate in cells grown in the presence of FCS. In fact, cholesterol-depleted cells (grown in the presence of LDS) showed an increase in the amount of lanosterol present (Fig. 4). These data do not suggest that demethylation reactions are suppressed in FCS-grown CHO cells. Thus, hepatic cells and nonhepatic cells appear to be regulated at different steps in the cholesterol biosynthetic pathway. Because HMG-CoA reductase is a regulatory enzyme in the cholesterol biosynthetic pathway (as confirmed in our cells [data not shown]) and its activity varies in response to the LDL present in the growth medium (16), the presence of an endogenous pool of squalene in these cells under noncholesterogenic conditions is somewhat surprising. Although we do not have data to explain this phenomenon, reductase activity may possibly be affected, in part, by the size of the endogenous pool of squalene. Future studies of the variations of this pool in relationship to reductase activity may shed light on this phenomenon.

REFERENCES

1. Rodwell, V.W., McNamara, D.J., and Shapiro, D.J. (1973) Adv. Enzymol. 38, 373-412.
2. Faust, J.R., Goldstein, J.L., and Brown, M.S. (1979) Proc. Natl. Acad. Sci. USA 76, 5018-5022.
3. Burns, P., Welshman, I.R., Edmond, J., and Spector, A.A. (1979) Biochim. Biophys. Acta 572, 345-351.
4. Chang, T.Y., and Limanek, J.S. (1980) J. Biol. Chem. 255, 7787-7795.
5. Astruc, M., Tabacik, C., Descomps, B., and Crastes de Penlet, A. (1977) Biochim. Biophys. Acta 487, 204-211.
6. Shechter, I., Fogelman, A.M., and Popjak, G. (1980) J. Lipid Res. 21, 277-283.
7. Gonzalez, R., Carlson, J.P., and Dempsey, M.E. (1979) Arch. Biochim. Biophys. 196, 574-580.
8. Popjak, G. (1969) Methods. Enzymol. 15, 393-454.
9. Douglas, T.J., and Paleg, L.G. (1972) Plant Physiol. 49, 417-420.
10. Caras, I.W., and Bloch, K. (1979) J. Biol. Chem. 254, 11816-11821.
11. Cham, B.E., and Knowels, B.R. (1976) J. Lipid Res. 17, 176-181.
12. Lowry, O.H., Roesbnrough, N.H., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
13. Goldstein, J.L., and Brown, M.S. (1977) Ann. Rev. Biochem. 46, 897-930.
14. Willett, J.D., Sharpless, K.B., van Tamelen, E.E., and Clayton, R.B. (1967) J. Biol. Chem. 242, 4182-4191.
15. Havel, C., Hansbury, E., Scallen, T.J., and Watson, J.A. (1979) J. Biol. Chem. 254, 9573-9582.
16. Brown, M.S., and Goldstein, J.L. (1980) J. Lipid Res. 21, 505-517.

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Side-Chain Structural Requirements for Sterol-Induced Regulation of *Phytophthora cactorum* Physiology

W. DAVID NES* and **ALLAN E. STAFFORD**, *Plant Physiology and Chemistry Research Unit, WRRC-ARS/U.S. Department of Agriculture, Berkeley, CA 94710*

ABSTRACT

The influence of cholesterol, (E)-17(20)-dehydrocholesterol, sitosterol, (Z)-17(20)-dehydrocholesterol and 20-isocholesterol on growth and sexual morphogenesis in *Phytophthora cactorum* has been examined. Optimal growth-response and production of oospores occurred with the 3 former sterols, which possessed "right-handed" side chains (C22 *trans*-oriented to C13). Abnormal hyphae and aborted oospores were evident in mycelia cultured with sterols having side chains with "left-handed" structures, i.e., 20-isocholesterol and (Z)-17(20)-dehydrocholesterol. The induction of the sexual cycle lacked a selectivity for stereochemistry in the side chain. The results are interpreted to imply that fungal recognition of the sterol molecule in the reproductive phase of the life cycle is of 2 types: one involves discrimination of stereochemical features of the sterol side chain (oospore production); in the other, no functional significance can be attributed to conformation or configuration of side chain moieties (sexual structure induction). Growth response to dietary sterol seems to fall into the former category.

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INTRODUCTION

The ultimate tetracyclic products, e.g., 4,4,14-tris-desmethylsterols, of 2,3-oxidosqualene cyclization that occur as membrane components, are sterols that possess a 20α-H atom and a side chain that presumably orients functionally as the right-handed skew conformer (1). Fungi (1-4), bacteria (5-7) and insects (8,9) with a nutritional dependence on polycyclic isopentenoids (steroids and triterpenoids) for growth and reproduction have been useful model systems to assess the similarities (and differences) in the biochemical and physiological roles of these molecules in otherwise evolutionarily divergent organisms. *Phytophthora cactorum*, which causes a collar rot of various crop roots, is especially attractive as an organism with which to explore the significance of sterol function. Despite the fungus's failure to epoxidize squalene (10,11), dietary sterols available from its host or the culture medium (4) are accumulated by mycelial membranes (12,13) affecting growth (14,15) and biochemical properties (3,16-18). Reproduction can also be "turned-on," resulting in the production of numerous oospores (19).

The purpose of the present communication is to report the effect of several sterols that are stereochemically modified, producing "right- and left-handed" side chains (1), for their ability to concurrently stimulate growth and induce oospores in *P. cactorum*. In order to form a basis for the structure-activity comparisons, detailed studies of uptake, derivatization and physiological parameters, with cholesterol as a standard, were made first. Previous investigations with the C20 stereoisomers of cholesterol in supporting growth of anaerobic yeast (20-22), metabolism by a protozoan (23), inhibition of hepatic cholesterol synthesis in a mammal (24) and lipid vessicle formation (25) demonstrated that the extent to which the side chain is recognized depends on the biological and physicochemical systems involved. In the present study we observed that the ability for the fungus to discriminate between the various synthetic and naturally occurring side chains is significant to the life cycle of the pathogen. In contrast, however, to inferences in the literature (26,27), we now find that the kinds of recognition of the sterol by *P. cactorum* are not as found in *Achyla*, a related Oomycete, in the induction of the sexual cycle. Alternatively, some similarities in the sterol requirements for membrane structure are implied.

*To whom correspondence should be addressed.

MATERIALS AND METHODS

Culture Methods

The test organism, *P. cactorum* (strain 51-22), obtained from the U. C. Berkeley fungal collection, was grown on a synthetic sucrose-asparagine medium as described by Elliott (26) and modified according to Nes et al. (14). The method for quantitating the various sexual reproductive structures has been described in the literature (19,26,30). Two stock cultures were routinely maintained at room temperature: one set was maintained on clarified V8 juice solidified by the addition of

Difco agar (20 g/l) and a second set was maintained on agar-supplemented synthetic medium. The only difference in the medium used for the experimental cultures with that of the second set was the addition of sterol dispersed in ethanol. Difco agar was recently shown to contain trace levels of cholesterol (28,29). The mycelia, originally cultured on the V8 juice–agar medium and producing an orange colored mat with no significant aerial hyphae, were transferred to a synthetic medium supplemented with agar. Every 2 weeks the fungus was transferred (5 mm plug) to fresh, agar-supplemented synthetic medium to which no additional sterol had been added. After ca. 2 transfers, the mycelia were white with aerial mycelium. This mat form served as the inoculum source for the various sterol supplementation experiments. When the mycelium was serially transferred to synthetic agar media (containing only trace sterol), the ability of the fungus to produce oospores in response to cholesterol supplementation diminished (4). Thus, every 6 months we initiate new synthetic, agar-supplemented stock cultures from the V8-cultures. Sterols (10 μg/ml of medium) were added as an ethanolic solution (10 μl or 2 μl/ml of medium, depending on the treatment) to the agar-supplemented synthetic media as the agar was solidifying. Ethanol at 2 μl/ml had no effect on growth or reproduction of cultures grown on agar. The higher level of ethanol (10 μl/ml) had no observable effect on reproduction or hyphal extension. However, as recently reported (31), dry wt of the fungus was increased by ca. 50% with 10 μl/ml ethanol. This ethanol effect was independent of the addition of sterol (within the concentration range tested) to the medium. No apparent synergistic or additive effects resulted from sterol-ethanol combinations. The effect that the greater amount of ethanol has on dry wt production may be related to the respiratory competency of the mitochondria analogous to that described in yeast (32). Analogous effects of high levels of ethanol (10 μl/10 ml of medium) supplied to mycelia cultured on liquid media are not apparent; in fact this level inhibits their growth (Poley and Nes, unpublished data). Also we have found that 0.5 μg of sterol/ml of medium is sufficient to stimulate maximal growth of mats cultured in synthetic liquid medium (Nes and Poley, unpublished data). We preferred to use 10 μg/ml of sterol in the present set of experiments because this level produced maximal oospores numbers. As the amount of sterol in the media decreases a corresponding decrease in the number of oospores is observed (Nes and Poley, unpublished data).

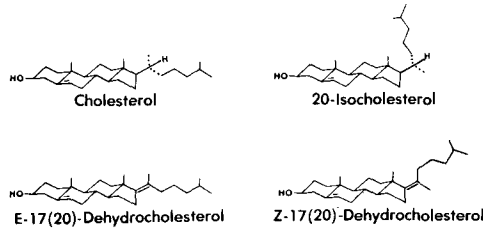

FIG. 1. Structures of some of the sterols incubated with *P. cactorum*.

Chemicals

[4-^{14}C] Cholesterol (59.4 mCi/m Mol) was purchased from New England Nuclear, Boston, MA. Cholesterol and sitosterol (24α-ethylcholesterol) were purchased from Applied Science Labs, State College, PA, and recrystallized from ethanol. The sitosterol contained a small percentage of 24-methylcholesterol. This contaminant was removed by chromatographing the commercial sitosterol on LH-20 Sephadex, developed isocratically with 5% MeOH in hexane. 20-Isocholesterol (also referred to as 20-epicholesterol), (Z)- and (E)-17(20)-dehydrocholesterol were the gifts of Dr. W. R. Nes. They had been synthesized according to established methods (33). Their structures are shown in Figure 1.

Lipid Extraction and Analysis of Sterols

The mycelia in each petri dish (5 dishes per structure-activity treatment) were recovered from the agar at each harvest (34). The mycelia were dried in vaccuo in an Abderhalden apparatus and then weighed, ground to a powder and extracted in a Soxhlet apparatus with refluxed acetone for 18 hr. In order to assess the derivatization of [^{14}C] cholesterol by the mycelia, the total lipid extract (TLE) from each harvest was chromatographed by TLC according to Nes et al. (34). Zones matching free sterols, sterylesters and sterylglycosides were scraped from the plate into scintillation vials containing POPOP cocktail (5 ml) and the radioactivity determined.

Sterols, reisolated from the fungus (without saponification) by thin layer chromatography (TLC) (34), were chromatographed on 3 packed gas liquid chromatographic (GLC) columns having different polarities. The retention times relative to cholesterol on 3% SE-30, 3% OV-17, and 1% SP-1000 packed columns (operated isothermally at 235°, 235° and 255°, respectively) for the 5 test sterols (≤ 99% pure by GLC) were: stiosterol–1.61, 1.68, 1.32; 20-isocholesterol–0.91, 0.89, 0.89; (E)-17(20)-dehydrocholesterol–0.93, 1.00, 0.98; and (Z)-

17(20)-dehydrocholesterol–0.87, 0.91, 0.92. We employed the 3 packed columns routinely because the EI-MS of the pairs, 20-isocholesterol and cholesterol, and (E)- and (Z)-17(20)-dehydrocholesterol are very similar (33). Furthermore, long-chain fatty alcohols were found to cochromatograph with the sterols on some TLC and GLC systems (29)–this can affect sterol quantitation. We used GLC-MS (GLC column–3% OV-17) to confirm the identities of the sterols found eluting in at least one GLC system used for routine analysis.

RESULTS AND DISCUSSION

Growth and Reproduction with Cholesterol

Although a great deal of information is available on sterol affecting both growth and reproduction of *P. cactorum* (for a recent review, cf. 19), the sequence of physiological events that result from the addition of cholesterol to the media has not been followed from the initiation of growth to the formation of oospores. Because we have been unable to produce oospores or oogonia in the liquid synthetic media over a 28-day incubation period (20° incubation temp) with 10 ppm of cholesterol, we chose to use the agar solidified synthetic media–a system previously shown to permit cholesterol to induce oospore production (28, 30). As shown in Figure 2, cholesterol initiates and maintains optimal growth, measured as changes in hyphal extension and dry weight. This "sparking" (40) of growth, in which the length of the lag phase is reduced by sterol supplementation, has recently been observed in yeast auxotrophic for sterols (40). The next event (Fig. 3) is the formation of the female sexual structures (oogonia), followed by fertilization and maturation of the latter into double-walled oospores. No consequential reproduction occurs in the absence of sterol (data not shown). In some of our experiments the control produces a few oogonia that variably mature into oospores. This, however, is attributable to the trace levels of sterol in the agar (29).

Derivatization of Cholesterol

Previous investigations (35,36) that examined sterol derivatization in *P. cactorum* were conducted under conditions in which no sexual reproduction occurred. In order to gain a more complete picture we examined this problem with cholesterol under culture conditions where multiple physiological processes could similarly be monitored. For the radiolabeled sterol experiment, [4-^{14}C]-cholesterol (3.6×10^6 cpm/1.8 mg cholesterol), as an ethanolic

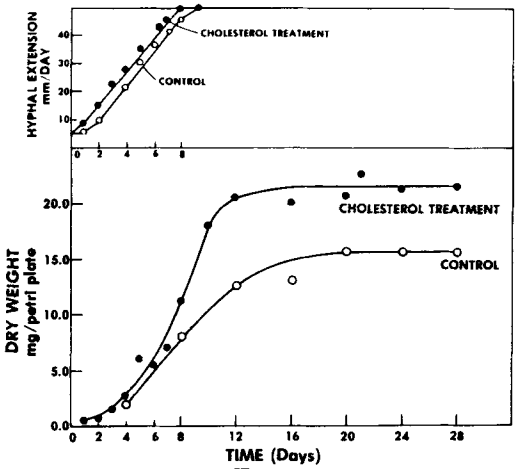

FIG. 2. Growth curves (top–growth measured as changes in hyphal extension; bottom–growth measured as changes in mycelial dry weight) of *P. cactorum* cultured on solid media at 20° in the dark.

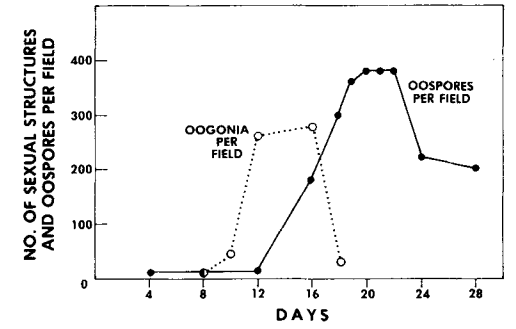

FIG. 3. Sequence of reproductive events exhibited by *P. cactorum* that can be readily observed microscopically and quantitated. Under these test conditions male sexual organs are not readily apparent, nor are the asexual zoospores.

solution, was distributed equally (5 ml aliquot) into each of 36 petri dishes, each of which contained 10 μg nonradioactive cholesterol and 2 μl ethanol per ml of medium. The first harvest (day one, post inoculation) contained the mycelia from 10 petri dishes. The second harvest (days 4, 6, 7-10, 14, 16, 20, 24, 28) contained mycelia from 2 petri dishes. Insufficient mass was available in the 24-hr period before the first harvest to obtain earlier data points. When the TLE of each of the mycelia harvested during the 28-day period was examined by TLC radioscanning and by comparing the radioactive counts in the 3 zones corresponding to free, esterified and glycosylated sterols, no significant differences in time were apparent in the ratio (ca. 72:26:2) of these molecular forms. In a separate experiment, cultures (ca. 5 per

treatment) with 10 ppm cholesterol were harvested at 12, 15, 21 and 28 days. The amount of free sterol in the mycelium was ca. .01% of the dry weight in each of the 4 harvests. Thus, no change occurred in the level of free or derivatized cholesterol in the mycelia during growth or reproduction. These findings contrast with data obtained with fungi capable of de novo sterol synthesis where, on entering the stationary phase, the sterylester pool greatly increases in relation to the free sterol pool (32). Furthermore, our results agree with Hendrix (37,38) and do not confirm the suggestion of Elliott and Knights (35) that derivatization of free sterols controls reproduction. In another experiment (data not shown) the TLE from the combined mycelia (5 petri dishes) of a cholesterol treatment (10 ppm) was chromatographed by TLC. The TLC plate was divided into 15, 1 cm zones and each zone scraped and the material eluted from the plate with ether. Each of these zones was bioassayed for oospore production. The only zone that produced significant oospores was the zone corresponding to 4,4-desmethyl sterols. Although we and others (39) observe the occurrence of steroid metabolites (distinct from the sterylglycosides and sterylesters, therefore presumably autooxidation products) from radiolabeled sterol feeds with *P. cactorum,* these compounds, as they occur in the mycelium, have no obvious biological activity. Had derivatization of the steroid pool been a major element in the reproductive cycle, we would have expected a significant change in the free sterol-to-derivatized pool ratio before or at the onset of oospore production.

Structure-Growth Relationships

Growth response to sterol supplementation was measured in 2 ways: changes in hyphal extension and dry weight. Growth and mycelial membrane biogenesis of filamentous fungi, e.g., *Phytophthora,* unlike single-celled organisms, e.g., yeasts, occur by localized extension of hyphal tips (41,42). The addition of 4 of the 5 test sterols to the culture medium affected both parameters of mycelial growth. Each of the 5 test compounds were recovered from stationary phase cultures and their identities confirmed by GLC and GLC-MS (mass spectroscopy). Cholesterol was present in every mycelial extract. To ensure that cholesterol was a contaminant in those cultures other than the cholesterol treatment and not the product of metabolism, e.g., reduction of the Δ17(20)-bond, we incubated another set of synthetic liquid cultures with 10 ppm of each of the sterols. These cultures were maintained for 2 weeks in 50 ml synthetic media/250 ml flasks and inoculated with a mycelial homogenate. The homogenate was formed from cultures that had been grown for 2 weeks on a sterol-free synthetic media and seeded with mycelial fragments. The GLC analysis of the 4-desmethyl zone from each mycelial extract failed to detect cholesterol, except in the cholesterol treatment. Thus, the inability of some sterols to effect a stimulatory growth-response in *P. cactorum,* e.g., (Z)-17(20)-dehydrocholesterol, cannot be caused by its lack of accumulation by the mycelium. Morphological abberations of 6-day-old cultures treated with 20-isocholesterol and Z-17(20)-dehydrocholesterol were observed in the extent of hypal branching; many hyphae grew as a single tube from the implant with no branch at all. The radial growth measurement for the 20-isocholesterol treatment is somewhat misleading because this response could be interpreted to imply a stimulatory effect; rather, we suggest, this compound has a deleterious effect on the fungus. Comparable rate of growth (hyphal extension) and mycelial morphology similar to the cholesterol treatment (43) was observed for (E)-17(20)-dehydrocholesterol and sitosterol. The addition of 3 of the 5 test sterols to the culture media stimulated growth as measured by the dry weight (Table 1), whereas 4 of the test sterols were stimulatory as measured by the radial growth. The results show that the initiation and maintenance of optimal growth (measured by both parameters) is accomplished by the 3 "right-handed" sterols.

Structure-Reproduction Relationships

From the time-course growth and reproductive studies with cholesterol treatment (Figs. 2 and 3), we observed that maximal oospore production occurred on the twenty-first day following the inoculations. Thus, all quantitative comparisons were made at this time. As shown in Table 1, all 5 test sterols induced oogonia formation. The naturally occurring 4-desmethyl sterols, e.g., cholesterol and fucosterol, when supplied to *Achlya* as an exogenous hormonal supplement, fail to induce sexual structure formation. Apparently only specific polyoxygenated steroids, e.g., antheridiol and oogoniol, derived by endogenous metabolism of fucosterol, stimulate the antheridia and oogonia to form (27 and ref. cited therein).

When sitosterol was bioassayed in *P. cactorum* more oospores were formed than with cholesterol or (E)-17(20)-dehydrolesterol. On

TABLE 1

The Effect of Sterols on Sexual Reproduction and Vegetative Growth in *Phytophthora cactorum*

Sterol added	Oogonia[a] Mean SD	Aborted oogonia Mean SD	Oogonia with oospores Mean SD	Total	Mean[b] 21-day dry weight (mg) Mean	Mean 6-day diameter (mm) Mean SD Rate
Cholesterol	0[b]	0	335 ± 43	335	30.1	42.0 ± 0.66 [6 mm/day]
20-Isocholesterol	150 ± 31	200 ± 67	10 ± 6	345	20.3	42.5 ± 1.02 [6.5 mm/day]
E-17(20)-Dehydrocholesterol	0[b]	0	313 ± 24	323	29.8	45.0 ± 0.66 [5.8 mm/day]
Z-17(20)-Dehydrocholesterol	60 ± 23	5 ± 5	0	65	22.8	37.0 ± 0.66 [6.0 mm/day]
24α-Ethylcholesterol	0	0	521 ± 51	521	30.0	43.0 ± 0.66 [6.0 mm]
Control (sterol-free)	5	0	0	5	21.0	36.0 ± 0.66 [6.0 mm/day]

[a]Mean (with standard deviation–SD) oogonia and oospore count with *P. cactorum* for 4 radial transerts in each of 5 petri plates. Mycelia were cultured for 3 weeks in the dark on agar at 20°. Sterols were added at a level of 10 μg/ml dissolved in warm ethanol at 2 μl/ml (v/v).

[b]The contents of 5 petri dishes were pooled into one flask and the dry weight obtained following the removal of the agar.

[c]The diameter was determined each day for 6 days; from this information the rate was calculated.

the other hand, 20-isocholesterol and E-17(20)-dehydrocholesterol failed to produce oospores. Because we observed oogonia and structures that appeared to be aborted oospores, i.e., hollow cells rather than double-walled structures, the "left-handed" sterols were probably recognized in a deleterious fashion by the fungal receptor sites at some point. That certain Δ5-sterols induce production of oogonia which fail to mature into oospores, we conclude that formation of oogonia and oospores are controlled by different biochemical component(s) and these may differ from those observed in other Oomycetes, e.g., *Achlya* (19).

In conclusion, the molecular requirements of structure demonstrated in this study for initiating and maintaining growth are similar to previous microbial studies with singled-celled organisms (22). However, unlike *Achyla* (44), where the induction of the female sex organs is influenced by the stereochemistry of the side chain (45), *P. cactorum* may use either of the "right- and left-handed" sterols for induction of the oogonia. The production of functional oospores, however, requires "right" handed sterols. Sterol derivatization appears to precede the formation of oogonia and no quantitative changes in the proportion of free sterol to esters or glycoside occur during oospore production. Interestingly, no steroid hormones have been implicated in oospore production, only in the induction of the male and female gametangia. Whereas the induction process may involve sterol feedback (19) on enzymes controlling the so-called "α-hormone" (46), oospore production itself appears to be regulated by the fitness of the sterol as a component of the maturing oogonia membranes, i.e., the developing double wall (28), rather than being metabolized to an oxygenated steroid hormone. The extent to which sterol-induced alterations in the mycelial membranes effect the oogonia maturation process requires further study.

ACKNOWLEDGMENT

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REFERENCES

1. Nes, W.R., and McKean, M.L. (1977) Biochemistry of Steroids and Other Isopentenoids, University Park Press, Baltimore, MD.
2. Bloch, K.E. (1983) Crit. Reviews Biochem. 14, 47-92.
3. Parks, L.W., McLean-Bowen, C., Bottema, C.K., Taylor, F.R., Gonzales, R., Jensen, B.W., and Ramp, J.R. (1982) Lipids 17, 187-196.

4. Nes, W.D., Saunders, G.A., and Heftmann, E. (1982) Lipids 17, 178-183.
5. Rottem, S., Pfendt, E.A., and Hayflick, L. (1971) J. Bacteriol. 105, 303-330.
6. Odriozola, J.M., Waitzkin, E., Smith, T.L., and Bloch, K. (1978) Proc. Natl. Acad. Sci. 75, 4107-4109.
7. Dahl, J.S., Dahl, C.E., and Bloch, K. (1980) Biochemistry 19, 1467-1472.
8. Kircher, H.W., and Gray, M.A. (1978) J. Insect Physiol. 24, 555-559.
9. Ritter, K.S., and Nes, W.R. (1981) J. Insect Physiol. 27, 419-424.
10. Gottlieb, d., Knaus, R.J., and Wood, S.G. (1978) Phytopathology 68, 1168-1169.
11. Wood, S.G., and Gottlieb, D. (1978) Biochem. J. 170, 355-363.
12. Gonzales, R.A., and Parks, L.W. (1981) Lipids 16, 384-388.
13. Langcake, P. (1974) Trans. Br. Mycol. Soc. 64, 55-65.
14. Nes, W.D., Patterson, G.W., and Bean, G.A. (1979) Lipids 14, 458-462.
15. Elliott, C.G. (1977) Adv. Microbiol. Physiol. 15, 121-173.
16. Schlosser, E., and Gottlieb, D. (1968) Arch. Microbiol. 61, 246.
17. Calderone, R.A., and Norman, C. (1976) Mycologia 68, 440-445.
18. Troast, R., and Norman, C. (1971) Proc. W. Va. Acad. Sci. 43, 17-24.
19. Nes, W.D. in Isopentenoids in Plants: Biochemistry and Function (Nes, W.D., Fuller, G., and Tsai, L., eds.) (1983) pp. 267-290, Marcel Dekker, NY.
20. Pinto, W.J., and Nes, W.R. (1983) J. Biol. Chem. 258, 4472-4476.
21. Nes, W.R., Sekula, B.C., Nes, W.D., and Adler, J.H. (1978) J. Biol. Chem. 253, 6218-6225.
22. Pinto, W.J., Lozano, R., Sekulà, B.C., and Nes, W.R. (1983) Biochem. Biophys. Res. Commun. 112, 47-54.
23. Nes, W.R., Joseph, J.M., Landrey, J.R., and Conner, R.L. (1978) J. Biol. Chem. 253, 2361-2367.
24. Erickson, K.A., and Nes, W.R. (1982) Proc. Natl. Acad. Sci. 79, 4873-4877.
25. Nes, W.R., Adler, J.H., Billheimer, J.T., Erickson, K.A., Joseph, J., Landrey, J.R., Marcaccio-Joseph, R., Ritter, K.S., and Conner, R.L. (1982) Lipids 17, 257-262.
26. Elliott, C.G. (1979) J. Gen. Microbiol. 42, 425-435.
27. McMorris, T.C. (1978) Lipids 13, 716-722.
28. Nes, W.D., and Nes, W.R. (1983) Experientia 39, 276-280.
29. Nes, W.D., and Stafford, A.E. (1983) Proc. Natl. Acad. Sci. 80, 3227-3231.
30. Nes, W.D., Patterson, G.W., and Bean, G.A. (1980) Plant Physiol. 66, 1008-1011.
31. Nes, W.D., Saunders, G.A., and Heftmann, E. (1983) Phytochemistry 22, 75-78.
32. Parks, L.W. (1978) Crit. Rev. Microbiol. 6, 301-341.
33. Nes, W.R., Varkey, T.E., Crump, D.R., and Gut, M. (1976) J. Org. Chem. 41, 3429-3433.
34. Nes, W.D., Saunders, G.A., and Heftmann, E. (1981) Lipids 16, 744-748.
35. Elliott, C.G., and Knights, B.A. (1981) Lipids 16, 142-145.
36. Elliott, C.G., and Knights, B.A. (1974) Biochim. Biophys. Acta. 360, 78-87.
37. Hendrix, J.W. (1975) Can. J. Microbiol. 21, 735-737.
38. Hendrix, J.W. (1975) Mycoligia 67, 663-666.
39. Elliott, C.G. (1983) in *Phytophthora:* Its Biology, Taxonomy, Ecology, and Pathology (Erwin, D.C., Bartnicki-Garcia, S. and Tso, P.H., eds.) pp. 71-80, American Phytopathological Society, St. Paul, MN.
40. Rodriquez, R.J., Taylor, F.R., and Parks, L.W. (1982) Biochem. Biophys. Res. Commun. 106, 435-441.
41. Meyer, R., Parish, R.W., and Hohl, H.R. (1976) Arch. Microbiol. 110, 215-224.
42. Robertson, N.F. (1965) in The Fungi, an Advanced Treatise (Ainsworth, G.C., and Sussman, A.S., eds.), Vol. I, pp. 613-623, Academic Press, NY.
43. Nes, W.D., Hanners, P.K., Bean, G.A., and Patterson, G.W. (1982) Phytopathology 72, 447-450.
44. Preus, M.W., and McMorris, T. JC. (1979) J. Am. Chem. Soc. 101, 3066-3071.
45. McMorris, T.C., Le, P.H., Preus, M.W., Schow, S.R., and Weihe, G.R. (1983) J. Am. Chem. Soc. 48, 3370-3372.
46. Ko, W.H. (1978) J. Gen. Microbiol. 107, 15-20.

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METHODS

Selective Oxidation of Steroidal Allylic Alcohols Using Pyrazole and Pyridinium Chlorochromate

EDWARD J. PARISH*, SARAWANEE CHITRAKORN, and **SUSAN LOWERY,** *Department of Chemistry, Auburn University, Auburn University, AL 36849*

ABSTRACT

This paper presents a modified method for the selective oxidation of allylic alcohols. Pyrazole, when used with pyridinium chlorochromate, is a mild and useful reagent system for the rapid and selective oxidation of steroidal allylic alcohols to the corresponding α, β-unsaturated ketones. The reaction of each substrate was carried out by adding the oxidant to a dry methylene chloride solution containing pyrazole and an allylic alcohol. This report is the first on the use of pyrazole to augment selective oxidation by a chromium (VI) reagent.
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INTRODUCTION

In many instances, the chemical synthesis of steroids of biological interest requires the controlled or selective oxidation of an allylic hydroxyl function on the steroid molecule. Moreover, this transformation is sometimes difficult to accomplish with a high yield when conventional reagents are used. We have undertaken the development of improved reagent systems to accomplish this reaction (1,2). We now wish to report that pyrazole, when used with the versatile oxidant, pyridinium chlorochromate, forms a convenient and useful reagent system for the rapid and selective oxidation of steroidal allylic alcohols. To our knowledge, this report is the first of the use of pyrazole to augment selective oxidation by a chromium (VI) reagent. Pyridinium chlorochromate (PCC) has recently become widely used in organic synthesis for the oxidation of primary and secondary alcohols to carbonyl compounds (1). This reagent, in methylene chloride containing pyridine or 3,5-dimethylpyrazole, was reported to effect the selective oxidation of the allylic hydroxyl function of a number of steroidal alcohols (2,3). In this report, we have endeavored to develop an improved reagent system to accomplish selective oxidations.

EXPERIMENTAL

General Methods

Melting points were determined with an electrothermal capillary apparatus and are uncorrected. Infrared (IR) spectra (KBr pellet) were recorded by a Perkin-Elmer Model 580 spectrometer. Proton nuclear magnetic resonance (NMR) spectra (deuterated chloroform solvent) were obtained with a Varian EM-390 spectrometer using tetramethylsilane as an internal standard. Proton chemical shifts (δ) for the C18 and C19 angular methyl resonances were calculated by the method of Zurcher (4). Ultraviolet (UV) spectra (ethanol solution) were recorded with a Cary 17 spectrometer. Mass spectral (MS) analyses were conducted using a DuPont 491 mass spectrometer. Gas liquid chromatographic (GLC) analyses were performed on a Varian 3700 gas chromatograph (GC) equipped with dual flame ionization detectors (FID) using 3% OV-1 and 3% OV-17 columns (270 C). Silanized glass columns (6 ft x 2 mm i.d.) were employed using nitrogen as the carrier gas (25 ml/min). Thin layer chromatography (TLC) was carried out on plates of Silica gel G (Analtech, Newark, DE) with the components visualized after being sprayed with molybdic acid (5). Solvent systems for TLC analysis were: 50% ethyl acetate in toluene, 50% ether in toluene and 75% ether in hexane. American Chemical Society solvents were used and methylene chloride was dried over molecular sieves (type 3A) before use. Column chromatography employed silica gel (60-200 mesh) on columns that were 60 cm x 1.5 cm. All isolated reaction products and starting materials were compared with authentic compounds (TLC and GLC, IR, NMR and MS) (2,3,6-10).

General Oxidation Procedure

Pyridinium chlorochromate (3 equivalents, 388 mg, 1.8 mmol) was added to a solution

*To whom correspondence should be addressed.

(50 ml) of the sterol (0.60 mmol) in a mixture of dry methylene chloride and pyrazole (2%, 14.7 mmol) at 2-3 C. After stirring for 30 min under nitrogen, a saturated NaCl solution was added and the mixture was thoroughly extracted with chloroform. The resulting extracts were dried over anhydrous magnesium sulfate, filtered and evaporated to dryness under reduced pressure to give a brown residue that was subjected to column chromatography using a solvent gradient of ether in toluene (compound 2 in Table 1 required a gradient of ether in hexane). The purified material was recrystallized from acetone and water to give the products shown in Table 1.

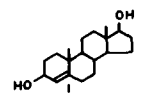
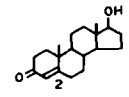
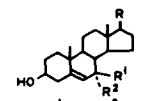
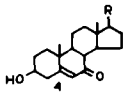
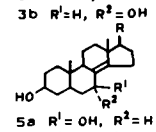
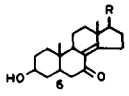
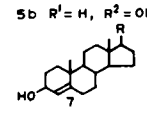
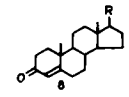
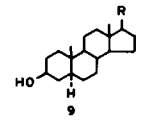
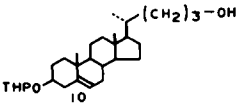
RESULTS AND DISCUSSION

The selectivity of this reagent was indicated by its failure to significantly oxidize saturated primary and secondary alcohols relative to allylic alcohols. Using 3.0 equivalents of pyridinium chlorochromate in methylene chloride containing an excess of pyrazole (2%) at 2-3 C, several steroidal allylic alcohols were successfully oxidized to the corresponding α, β-unsaturated carbonyl compounds (Table 1). The allylic hydroxyl function of **1**, **3a**, **5a** and **7** is in the quasiequitorial configuration that makes them more amenable to chromate oxidation. Quasiaxial allylic alcohols have been reported to have a slower rate of oxidation (11). The quasiaxial substrate **3b** gave a 92% yield of 7-keto sterol **4**, demonstrating the ability of this reagent to selectively oxidize allylic alcohols in either configuration. The attempted oxidation of **5b** (quasiaxial allylic alcohol) resulted in the formation of a complex mixture of oxidation products (observed by TLC); a 51% yield of the 7-keto sterol **6** was obtained. These results were a marked improvement over those observed when **5b** was oxidized by pyridinium chlorochromate in a 2% pyridine or 3,5-dimethylpyrazole solution of methylene chloride (2,3). Undesired side reactions have been reported by other workers during the chromate oxidation of allylic alcohols (11). In addition, **5b** and its esters have been reported to rearrange during treatment with acid, Oppenauer oxidation and pyrolysis (12,13). Treatment of alcohols **9** and **10** under identical conditions resulted in a greater than 90% recovery of starting material.

Manganese dioxide is commonly used for the selective oxidation of allylic and benzylic alcohols, but undesired side reactions, long reaction times and difficulty during the oxidation of hindered alcohols have been reported (11,14,15). Other chromate oxidizing reagents (16,17,18) and 2,3-dichloro-5,6-dicyanobenzoquinone (14,19) have been reported to selectively oxidize benzylic and allylic alcohols. In general, these reactions are characterized by moderate selectivity and extended reaction times. The observed selective oxidations of steroidal allylic alcohols by pyridinium chlorochromate and pyrazole (under basic conditions) at low temperatures, and the ease of using this procedure offers a useful and attractive alternative to other reagents in oxidations of complex allylic alcohols.

TABLE 1

Selective Oxidation of Steroidal Allylic Alcohols with Pyrazole and Pyridinium Chlorochromate and Pyrazol

Alcohol[a]	Ketone product	Percentage of yield[b] (% recovered starting material)
		88(–)
 3a R^1=OH, R^2=H 3b R^1=H, R^2=OH		93(–) 92(–)
 5a R^1=OH, R^2=H 5b R^1=H, R^2=OH		90(–) 51(11)
		93(–)
		–(92)
		–(93)

[a]The reactions were carried out in dichloromethane at 2-3 C using 0.6 mmol of alcohol.

[b]Yield of isolated ketone product. Values deleted indicate that no significant amount of the indicated compound was isolated from the reaction mixture.

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REFERENCES

1. G. Piancatelli, A., Scettri, A., and D'suria, M. (1982) Synthesis 245-258.
2. Parish, E.J., and Schroepfer, G.J., Jr. (1980) Chem. Phys. Lipids 27, 281-288.
3. Parish, E.J., and Scott, A.D. (1983) J. Org. Chem. 48, 4766-4668.
4. Zurcher, R.F. (1963) Helv. Chim. Acta 46, 2054-2088.
5. Knapp, F.F., Jr., and Schroepfer, G.J., Jr. (1975) Steroids 26, 339-357.
6. Ruzicks, L., and Wettesein, A. (1935) Helv. Chem. 18, 1264-1275.
7. Bergstron, S., and Wintersteiner, O., (1941) J. Biol. Chem. 141, 597-610.
8. Tsuda, M., Parish, E.J., and Schroepfer, G.J., Jr. (1979). J. Org. Chem., 44, 1282-1284.
9. Fieser, L.F., Nakanishi, K., and Huang, W.-Y. (1953) J. Am. Chem. Soc. 75, 4719-4725.
10. Fieser, L.F. (1953) J. Am. Chem. Soc. 75, 5421-5422.
11. House, H.O. (1972) Modern Synthetic Reactions, pp. 265-267, Benjamin, Inc., Menlo Park, CA.
12. Fieser, LF., and Ourisson, G. (1953) J. Am. Chem. Soc. 75, 4404-4414.
13. Lutsky, B.N., Martin, J.S., and Schroepfer, G.J., Jr. (1971) J. Biol. Chem. 246, 6737-6744.
14. Fried, J., and Edwards, J.A. (1972) Organic Reactions in Steroid Chemistry, Vol. 1, p. 244, Van Nostrand Reinhold Co., NY.
15. Fatiadi, A.J. (1976) Synthesis 65-104.
16. Sananiello, E., and Ferraboschi, P. (1980) Synth. Commun. 10, 75-81.
17. Guziec, F.S., Jr., and Luzzio, F.A. (1982), J. Org. Chem 47, 1787-1789.
18. Huang, X., and Chan, C.-C. (1982), Synthesis, 1091.
19. Burn, D., Petrown, V., and Weston, G.O. (1960) Tetrahedron Letters L9, 14-15.

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Determination of the Composition of Mixed Micelles of Bile Salts by Kinetic Dialysis

M. LAKE and **D.T. ORGANISCIAK***, *Department of Biological Chemistry, School of Medicine, College of Science & Engineering, Wright State University, Dayton, OH 45435*

ABSTRACT

A kinetic dialysis method for determining the critical micelle concentration and the composition of mixed micelles has been adapted to study a binary system of bile acids. Using kinetic dialysis, the critical micelle concentrations of taurocholate, deoxycholate and taurodeoxycholate were determined. Monomer analysis was performed by colorimetric and radioactive tracer techniques on aliquots of the dialysates. Similarly, the composition of mixed micelles made from various molar fractions of deoxycholate and taurocholate containing tritium and ^{14}C tracers were determined by this method. The results suggest that, for these bile acids, mixed micelle composition is largely predicted by the molar composition of the binary system. Kinetic dialysis has proven to be a rapid procedure and to yield results that agree with critical micelle concentration values previously reported.
Lipids 19:553-557, 1984.

INTRODUCTION

The interaction of bile acids and lipids to form mixed micelles during digestion is important for the proper emulsification and absorption of lipophilic nutrients. Although a variety of methods are available for determining the critical micelle concentration (CMC) of single amphiphiles, few techniques are suitable for studying monomer concentrations in mixed micelle systems. Surface tension measurements that have been used to determine mixed micelle composition do not apply in a number of cases. For example, micellar systems that contain multiple ionic surfactants, in the absence or presence of small amounts of salt, and systems in which the solubility is close to the CMC, have been refractory to surface tension measurements (1). Bile salts that form dimers over a wide concentration range well above the CMC (2) have also been difficult to study in mixed micelles.

Recently, ultrafiltration has been used to determine the composition of mixed micelles (3). A variation of the filtration approach is kinetic dialysis, which separates monomers from micelles with of membrane of appropriate pore size. Kinetic dialysis measures the rate at which monomers diffuse through a membrane, and using a dialysis equation (4), the monomer concentration at t_0 can be calculated. The time required for kinetic dialysis measurements is on the order of 1-2 hr in contrast to equilibrium dialysis or Sephadex bead inclusion methods (5), which require up to 48 hr for analysis. Kinetic dialysis has previously been used to determine the CMC and mixed micelle composition of soap solutions (6,7), which have relatively high CMC values.

In the following study, kinetic dialysis was investigated as a method for determining the CMC of 3 bile acids: deoxycholate (DC), taurocholate (TC) and taurodeoxycholate (TDC). We also report the application of kinetic dialysis to the analysis of monomer content in a binary system containing radioactive bile salts and the determination of the molar fractions of each in the mixed micelle.

*To whom correspondence should be addressed.

MATERIALS AND METHODS

Dialysis Cell

A dialysis cell was made from 2 blocks of acrylic plastic (9 cm × 9 cm × 1.2 cm), with each block containing a circular cavity 6.4 cm diameter by 0.9 cm deep (ca. 30 ml vol). Two, 1 cm-wide circular rubber gaskets, lightly greased with silicone grease, were used to seal the dialysis membrane in place. A 4 mm hole was drilled in the top of each block for filling and removing of solution. Dialysis membrane tubing (MW cutoff of 1000) was obtained from Fisher Scientific, Inc. The tubing was cut, washed in deionized water and opened to fit between the 2 cavities. The 2 blocks were securely clamped and mounted on a mechanical shaker.

Procedure for CMC Determination

Solutions of TC (2 mM–10 mM) and DC or TDC (1 mM–5 mM) were prepared in 20 mM

sodium phosphate buffer, pH 7.4. The sodium salts of the bile acids were obtained from Calbiochem, La Jolla, CA. NaCl was added to give a final Na concentration of 156 mM. The solutions were brought to equilibrium overnight and 25 ml vol were dialyzed at 20 C against an equivalent sodium phosphate buffer. A smaller volume of solution than the cell capacity was used for proper agitation during dialysis. The shaker was operated at 100 RPM during dialysis and at various time intervals (1 and 2 hr), 0.5 ml aliquots were removed for analysis.

TC concentration in the dialyzate was determined by a modification of the Pettenkoffer reaction. To 0.5 ml of dialyzate was added 1.5 ml of distilled water, 2.0 ml of 0.4% (w/v) furfuraldehyde (recently distilled) and 2.0 ml of cold concentrated sulfuric acid. The solution was kept on ice as the acid was added. The contents were mixed and placed in a water bath at 65 C for 13 minutes. The solutions were cooled to room temperature and the color stabilized by adding 4.0 ml of glacial acetic acid. The contents were mixed and absorbance at 610 nm measured against a reagent blank.

Deoxy bile acids (DC and TDC) were determined by a separate colorimetric procedure. Distilled water (0.5 ml) was added to 0.5 ml of dialyzate followed by 4.0 ml of cold reagent, which was prepared by adding 1 g of p-dimethylamino-benzaldehyde to a mixture of 60 ml of concentrated H_2SO_4, 40 ml of H_3PO_4 and 10 ml of distilled water. Samples were then mixed and heated in a water bath at 65 C for 13 min. The solutions were cooled to room temperature and the absorbance determined at 425 nm against a reagent blank. The dialyzate concentration was determined as before, from standards run concurrently. Using these colorimetric procedures, TC could be determined in the presence of the other bile salts. However, the colorimetric procedure was not applicable for determining the concentration of the deoxy bile salts in the presence of TC.

Radioactive Isotope Procedure

Various concentrations of DC and TC containing tracer quantities of G-^{3}H DC (4.0 Ci/mmol) or 24-^{14}C TC (40.3 mCi/mmol) (New England Nuclear, Boston, MA) were prepared and dialyzed as described. From the determination of radioactivity in the starting solutions and the sample aliquots, the concentration of bile salt in the dialyzate was determined. For each bile salt a plot of dialyzate concentration vs the total concentration of the individual bile salt was made, at 1 and 2 hr time intervals.

In addition, mixed systems containing various molar fractions of DC and TC at a total bile salt concentration of 5 mM were prepared with labeled isotopes and dialyzed as before. Liquid scintillation counting procedures were used to measure ^{14}C, ^{3}H or ^{3}H/^{14}C.

The CMC values were calculated using the dialysis equation (4) CMC (mM) = $a^2/(a\text{-}b/2)$, where a is the concentration of bile salt in the dialyzate at time t; b is the concentration in the dialyzate at 2t.

RESULTS AND DISCUSSION

As illustrated in Figure 1, the rate of dialysis of TC monomer is nearly linear for the first 60 min and then decreases. Using the kinetic dialysis equation for CMC determinations, experimental time point 2t should be taken in the nonlinear region of the dialysis curve. As an example, for TC, if measurements are made at time points during the linear portion of the curve, the denominator (a-b/2) of the dialysis equation approaches zero and the CMC becomes erroneously large. On the other hand, if t and 2t are taken after the micellar system has attained equilibrium, the CMC determined will be erroneously small. Kinetic dialysis, therefore, is based on measurements taken during the period where the change in the rate of diffusion is nonlinear. In this study, CMC values were calculated from experimental time points taken after 1 and 2 hr, although nearly identical results were obtained for the time periods 45-90 min and 50-100 min of dialysis (Fig. 1). Optimal sampling times were also determined for each set of

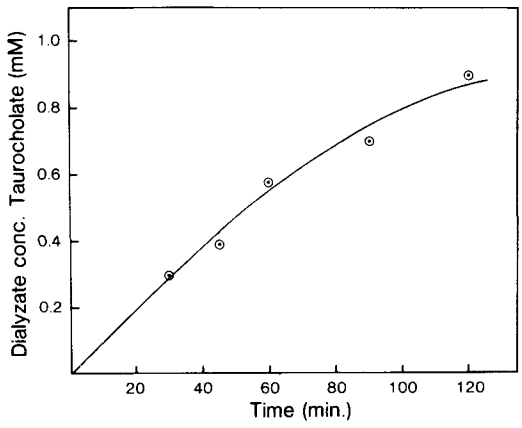

FIG. 1. Time dependence of TC dialysis. A 10 mM solution of TC was prepared and dialyzed against buffer. Aliquots were withdrawn periodically and analyzed colorimetrically as described in Materials and Methods.

experimental conditions. A comparison of our findings to published results (see below) suggests that for micelles with an unknown CMC reliable measurements can be made from determinations based on the diffusion rate, as in Figure 1.

The time required for dialysis also depends on the pore size of the membrane used. A membrane must be selected on the basis of micelle aggregation number and whether or not dimers form, as is the case for bile salts. Because TC and TDC have dimer molecular weight of over 1000 daltons, a dialysis membrane with a 1000 MW cutoff was chosen. The dimer MW of DC is slightly less than 1000 but the CMC determined for DC agrees reasonably well with literature values (9, see also Table 1).

Table 1 contains the CMC values for 5 mM concentrations of bile salts determined colorimetrically and by the isotope procedure. The CMC for TC whose monomer concentration varies according to the total concentration, even above the CMC, (5) was determined at 5 and 10 mM concentrations. A comparison of experimental and literature values (column 3) indicates that nearly identical results were obtained by either analytical procedure for monomer analysis. Despite the general agreement between the experimental and literature values, the colorimetric method of analysis is subject to error because of the difficulty of controlling temperature during the color development step. The accuracy in using the radioactive bile salts was improved when the dialyzate concentrations were corrected for nonspecific radioactive contamination.

Preliminary analysis revealed that radioactive contamination of either ^{3}H-DC or ^{14}C-TC solutions during the dialysis procedure gave CMC values about twice those reported (8) and higher than the colorimetric procedure. Thus, a correction factor was made for each bile salt, which was based on extrapolation of dialyzate concentration to zero bile salt concentration as shown in Figure 2 for DC. The Y intercept values for the 1 and 2 hr dialysis times were then subtracted from the corresponding dialysis concentrations and the CMC subsequently determined. These radioactive contaminates, which did not react colorimetrically and were not removed by TLC of radioactive bile salts, may result from degradation of bile salts during the overnight equilibration that preceeded dialysis. The Sephadex bead technique of Ammon et al. (5), which also employed radioactive bile salts, gave CMC values ca. twice the values reported here. Although the CMC values reported for bile salts vary widely, we chose to compare our results with those reported from a thorough study under various conditions (8).

The change in monomer concentrations for a 5 mM binary micelle system containing radiolabeled TC and DC is shown in Figure 3 By either the colorimetric or radioistope procedure, the CMC of TC increases as a function of the increase in the molar fraction of TC in the original solution. Similarly, the CMC of DC also increases as a function of the molar percentage of DC, but the effect appears to be linear. The CMC for pure TC at the 5 mM concentration used in the experiments was 2.1 mM as opposed to 2.8 mM for the 10 mM solution of pure TC (see Table 1). The nonideal behavior of some bile salts (e.g., TC) results in an increasing monomer concentration as a function of total concentration above the CMC (5). The reason for this effect is not entirely clear. Thus, for the bile acids, the conditions at which the CMC is determined must be carefully stated for comparative purposes.

Table 2 compares the molar fraction of TC, in mixed micelles of TC and DC, with the total molar fraction of TC at various molar ratios. The molar fraction of TC was determined by subtracting the monomer concentration (Fig. 3) from the total concentration of equivalent bile salt and dividing the TC micelle concen-

TABLE 1

CMC Values of Various Bile Salts Determined by Kinetic Dialysis[a]

Bile salt	Colorimetric procedure (mM)	Isotope procedure (mM)	Literature (9) (mM)
Deoxycholate	1.4 ± 0.4	1.2 ± 0.2	1.0
Taurocholate (5 mM)[b]	2.1 ± 0.4	1.8 ± 0.3	—
Taurocholate (10 mM)	2.8 ± 0.3	2.8 ± 0.3	2.7
Taurodeoxycholate	0.8 ± 0.2	—	0.8

[a]Values represent the mean ± SD for n=4 determinations in parenthesis for 5 mM solution of pure bile acid.

[b]TC concentration determined at 5 mM and 10 mM.

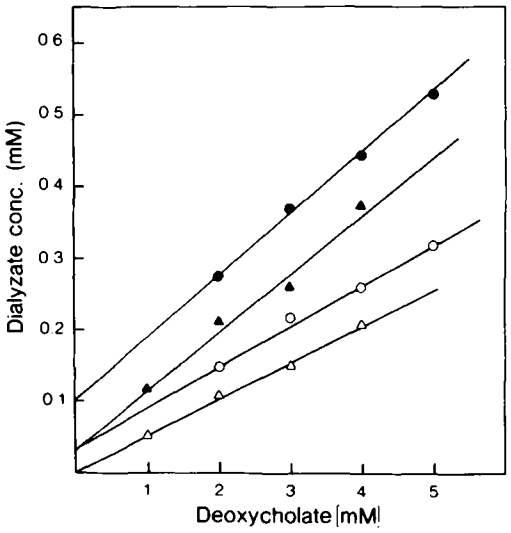

FIG. 2. Plot of monomer concentration in dialyzate at 1 and 2 hr intervals vs bile salt concentration for DC. Curves –○– and –●– are for pure DC, 1 and 2 hr, respectively; curves –△– and –▲– are for the DC-TC mixed system, 1 and 2 hr respectively.

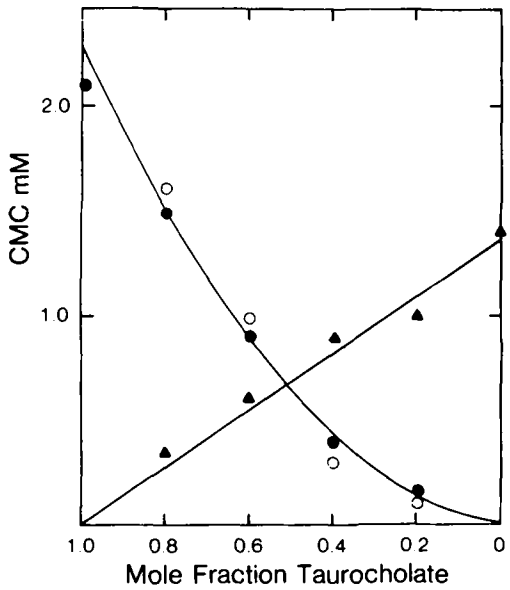

FIG. 3. Concentration of DC and TC monomers at various molar fractions: TC (–○–) determined colorimetrically; TC (–●–); DC (–▲–) determined by radioactive counting procedures. Nonspecific binding of DC and TC to membranes was less than 2% and 1% of the radioactivity used in these studies.

TABLE 2

Mole Fraction of Taurocholate in Mixed Micelles of Taurocholate and Deoxycholate[a]

Starting Concentration of TC/DC (mM)	Micelle concentration of TC	Micelle concentration of DC	Molar fraction TC	
			Prepared	Calculated
4.0/1.0	2.50	0.70	0.80	0.78
3.0/2.0	2.10	1.45	0.60	0.59
2.0/3.0	1.60	2.18	0.40	0.42
1.0/4.0	0.83	2.90	0.20	0.22

[a]Micelle concentrations were calculated by subtracting the monomer concentration from the total concentration for each bile salt (total concentration, 5 mM).

tration by the sum of the micelle concentrations of TC and DC. For TC, the micelle concentration varied from 62% to 83% of the concentration of TC in the starting solutions. For DC, however, the micelle concentration was a nearly constant 70-72% of the DC content in the prepared solutions. As shown in columns 4 and 5 of Table 2, the calculated molar fractions of TC in the micelles agrees very well with the molar fraction of TC in the original solutions. This result is not unexpected because the structures of both TC and DC are similar and the CMC values (at 5 mM) are not too different (2.1 vs 1.4 mM), indicating that the mixed micelle composition of TC and DC is determined primarily by statistical considerations (molar concentrations) in solution. The use of ^{3}H and ^{14}C labeled bile salts permits rapid analysis of a binary system. More complex systems of bile salts may be amenable to analysis with the use of high pressure liquid chromatography for separation and quantitation.

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REFERENCES

1. Funasaki, N., and Hada, S. (1979) J. Phys. Chem. 83, 2471-2475.
2. Nair, P.P., and Kritchevsky, D. (1971) The Bile Acids, Vol. 1, p. 319, Plenum Press, NY.
3. Warr, G.G., Grieser, F., and Healy, T.W. (1983) J. Phys. Chem. 87, 1220-1223.
4. Lake, M., and Rappaport, G. (1980) Rubber Chem. and Tech. 53, 1006-1012.
5. Ammon, H.V., and Walter, L.G. (1982) Anal. Chem. 54, 2079-2082.
6. Lake, M. (1982) Makromol. Chem. 183, 459-463.
7. Lake, M. (1983) J. Colloid Interface Sci. 90, 496-499.
8. Carey, M.C., and Small, D.M. (1969) J. Colloid Interface Sci. 31, 382-396.
9. Nair, P.P., and Kritchevsky, D. (1971) The Bile Acids, Vol. 1, pp. 303-308, Plenum Press, NY.

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Analytic Ultracentrifuge Calibration and Determination of Lipoprotein-Specific Refractive Increments[1]

TALWINDER S. KAHLON,[a] GERALD L. ADAMSON,[a] LAURA A. GLINES,[a] FRANK T. LINDGREN,[a]* MARIE A. LASKARIS[b] and VIRGIE G. SHORE,[b] [a]*Donner Laboratory, Lawrence Berkeley Laboratory, University of California, Berkeley, CA 94720 and* [b]*Lawrence Livermore Laboratory, Livermore, CA 94550*

ABSTRACT

Accurate quantification of the major classes and subfractions of human serum lipoproteins is an important analytical need in the characterization and evaluation of therapy of lipid and lipoprotein abnormalities. For calibrating the analytic ultracentrifuge (AnUC), we routinely use a Beckman calibration wedge cell with parallel scribed lines 1 cm apart. Such a cell gives a rectangular pattern in the schlieren diagram, which determines magnification and also provides an area corresponding to an invariant refractive increment. We have independently validated this wedge calibration cell using a special boundary-forming cell in which 1.174% sucrose is overlayered with distilled water. Comparing wedge cell area with extrapolated zero time boundary area refractive increment gives agreement to within less than 1%, corresponding to a refractive increment error of ±0.00002 Δn. Complete calibration for AnUC analysis of lipoproteins also requires accurate determination of the specific refractive increments (SRI) of the major lipoprotein classes, namely low density lipoprotein (LDL) and high density lipoprotein (HDL). These are measured in the density in which they are analyzed, i.e., 1.061 g/ml for LDL and 1.200 g/ml for HDL. Five fresh serum samples were fractionated for total LDL and total HDL and their SRI determined. Total lipoprotein mass was determined using precise CHN elemental analysis and compositional analyses. The results yielded corrected SRI of 0.00142 and 0.00135 Δn/g/100 ml for LDL and HDL. Thus, our current values using 0.00154 and 0.00149 Δn/g/100 ml underestimate LDL and HDL by 9% and 11%. Corrections of all previous LDL and HDL AnUC data can be made using appropriate factors of 1.087 and 1.106.
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INTRODUCTION

Human plasma lipoprotein distribution profiles have been analyzed quantitatively for ca. 35 years using the AnUC. However, information on calibration of the schlieren optical system is limited to the Beckman technical bulletin (1), which describes a special scribed quartz wedge calibration cell. No independent, accurate validation of this invariant means of checking the relative calibration of an AnUC appears to have been done. In addition, a reevaluation of the SRI for each major lipoprotein class is also necessary for accurate concentration determinations. The available lipoprotein SRI data are limited to a value of 0.00171 Δn/g/100 ml for β-lipoprotein (equivalent to LDL, S_f 0-12) and 0.00178 Δn/g/100 ml for α_1 lipoprotein, both measured in saline (2), to values of 0.00151 Δn/g/100 ml for S_f 0-12 and S_f 0-100 LDL measured in 1.063 g/ml NaCl (3) and to a value of 0.00158 Δn/g/100 ml for S_f 20-400 very low density lipoproteins (VLDL) as measured in 0.194 molal NaCl (4). These increments were measured at 5461 Å, similar to that with the Hg light source and filter in the AnUC. The latter VLDL SRI were measured with a precision Abbe refractometer using a $Na_{D\ 1,2}$ light source (5890 and 5896 Å). No experimental SRI data are available for HDL under the conditions of AnUC flotation in NaBr at d = 1.200 g/ml.

MATERIALS AND METHODS

In order to check the Beckman calibration cell (Part No. 306386) independently, a synthetic boundary cell was made from a double sector (2½°) Al-filled epoxy centerpiece by scribing 0.001″ deep scratches across the center partition on both faces. A 1.174% sucrose solution was introduced into one sector to a level ca. 3 mm below the scribe line. The other sector was then completely filled with distilled H_2O. After loading the rotor, the Model E ultracentrifuge (Beckman Instruments, Palo Alto, CA) was simultaneously pumped down and accelerated to 10,000 rpm. Under these conditions, at 26 C, a sharp sucrose boundary was formed at ca. 5000 rpm, and pictures were taken at 0, 2, 6, 8, 14, 22 and 30 min after reaching full speed (10,000 rpm). A standard Model E Beckman schlieren optical system equipped with a phase-plate wire combination,

*To whom correspondence should be addressed.
[1] A preliminary part of this study was presented at the 74th Annual AOCS meeting, Chicago, 1983.

set at 53°, was used (5). A pressure difference occurred of ~3 atmospheres between the 2 sectors, but this would have little effect on boundary stability as full speed during the run was 10,000 ± 20 rpm. To further validate this calibration without pressure differences, our centerpiece was scribed across the top between the 2 sectors. Sucrose boundary stability and total Δn of the boundaries before and after this scribing were indistinguishable.

Rectangular and gaussian schlieren patterns for both the standard calibration wedge and the sucrose boundary cell pictures were traced at a total magnification of 11.62 × with an enlarger (Omega, type B4, Simmons Brothers Inc., Long Island City, NY). Area of the tracings were measured using a sonic digitizer-minicomputer (6) and the changing sucrose boundary areas were extrapolated to zero time. The latter area represents the refractive increment of the 1.174% sucrose solution above distilled H_2O as measured by the schlieren optics of the ultracentrigue using an Hg source (5461 Å). A Bausch and Lomb Precision Abbe Sugar Refractometer (6), equipped with both Na_D and Hg (5461 Å) light sources, provided the refractive index difference between the 1.174% sucrose solution and the distilled water. Standard error of measurement for total Δn was $\pm 2 \times 10^{-5}$ Δn for both light sources (n = 10 independent samples read by 3 technicians). Serum samples that were very low in VLDL were fractionated for total LDL and total HDL (7). Such LDL and HDL samples were dialyzed 3 times for 18 hr against 1.061 g/ml and 1.200 g/ml densities NaBr containing 0.194 molal NaCl, respectively, at 4 C to remove all small organic molecules, e.g., glucose, amino acids and so forth. The solutions used for dialysis were adjusted to pH 7.4 by adding small amounts of 0.1 M NaOH or 0.1 M HCl. After dialysis, lipoprotein aliquots and the final dialysate were measured by precision refractometry and AnUC as described above for the sucrose solution, except the rotor speed was 52,640 rpm. However, the standard error of Δn measurement was significantly higher, $\pm 3 \times 10^{-5}$ Δn for LDL fractions and $\pm 5 \times 10^{-5}$ Δn for HDL fractions (n = 14 duplicate pairs each as read by 2 technicians). This greater error involves greater difficulty of reading small samples (0.02 ml) where the drop size, using the reflection reading, increases errors in n because of evaporation in samples containing higher salt concentrations.

Total lipoprotein mass was measured by CHN elemental analysis (5) of 200-300 μg samples. Protein content of these lipoprotein solutions was determined by the method of Lowry et al. (8) with bovine serum albumin as the standard and calibration by amino acid analysis of reference lipoprotein samples. Cholesteryl esters, free cholesterol, triacylglycerol and phospholipids were determined spectrophotometrically as described earlier (9). Human albumin present in the HDL subnatant was determined by radial immunodiffusion.

RESULTS AND DISCUSSION

Figure 1 shows schematically the comparison of the calibration procedures with results obtained by the Beckman calibration cell and by the special boundary-forming cell. At 11.62 fold magnification, the parameters of the calibration cell gave an area of 115.77 cm^2, corresponding to a Δn of 0.007638 as shown in the formulae in Figure 1. The extrapolated 0-time area for the 1.174% sucrose boundary at 11.62 × was 25.94 cm^2, corresponding to a Δn of 0.001711. The Δn by precision refractometry at 26 C for the sucrose solution minus the distilled water reading was 0.001717, and both Na_D and Hg source measurements were within the error of measurement. Thus, the 2 independent procedures agree to within 1%, which corresponds to the error of measurement of the precision refractometry for such samples, namely, ±0.00002 Δn.

Table 1 shows the mass analysis for total LDL and HDL fractions from 5 different subjects and their respective subnatant backgrounds. Similar data are also given for 2 frozen samples after 7 weeks and 1 year at −70 C. The −70 C freeze-thawed samples were selected to evaluate the potential for long-term frozen serum as a stable LDL and HDL standard. These data suggest that little change occurs in total macromolecular mass in both the LDL and HDL fractions and subnatants as the result

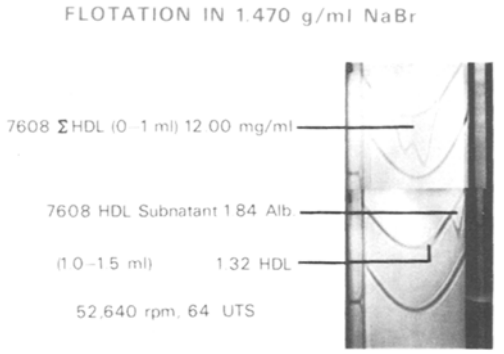

FIG. 1. Schematic comparison of refractive increment calibration by the Beckman scribed optical wedge cell and the sucrose boundary-forming cell.

TABLE 1

Total Lipoprotein Mass by CHN Elemental Analysis and Composition Analysis (mg/ml)

Sample #	% S_f 0-12	(LDL)[c] $2VL_1^0$	$4VL_2^1$	(HDL)[c] $2VLH_1^0$	$4VLH_{1.5}^1$
6569	96.2%	4.19 (4.03)[a]	0.29 (0.40)	8.00 (7.72)	0.58 (----)
6661	93.4%	4.47 (4.15)	0.21 (0.28)	8.38 (7.34)	0.53 (0.26)
6662	95.5%	5.18 (4.81)	0.29 (0.46)	8.85 (8.40)	0.79 (0.73)
7608	95.5%	8.42 (8.05)	0.86 (0.89)	12.00 (11.93)	3.16 (3.38)
7684	97.5%	10.65 (10.68)	1.31 (----)	13.25 (13.50)	3.05 (----)
Mean difference (5)		(95.6%)		(96.1%)	
7072 (frozen 7 wk)[b]	98.7%	9.37 (8.43)	1.32 (----)	12.94 (12.86)	3.41 (----)
7609 (frozen 1 yr)[b]	96.9%	8.73 (8.23)	1.10 (1.05)	14.23 (13.18)	3.11 (3.39)
Mean difference (2)		(92.1%)		(96.0%)	

[a]Values in parentheses are composition analysis, i.e., total protein, triglycerides, free cholesterol, cholesteryl esters, and P-containing phospholipids. No carbohydrates are analyzed nor are the 5-6% of lipids (free fatty acids and certain phospholipids) analyzed.

[b]Frozen sample at −70 C obtained earlier from subject 7608.

[c]$2VL_1^0$ and $2VLH_1^0$ are abbreviations for total low density and total high density dialyzed fractions, respectively, concentrated two-fold over serum. The corresponding LDL and HDL subnatant fractions, concentrated four-fold, are designated $4VL_2^1$ and $4VLH_{1.5}^1$, respectively. The variations in the subnatant macromolecular mass reflect largely individual variations which, in part, may explain the differences in SRI's obtained by schlieren analysis and refractometry.

of −70 C storage and single thawing over a year period. Thus, frozen aliquots of such a sample with very little VLDL might provide an acceptable lipoprotein frozen standard for LDL and HDL, as suggested earlier (6). Comparison of total mass by elemental CHN analysis with chemical composition analyses of the LDL and HDL fractions indicates 4% lower values for the latter. This is very good agreement because composition analyses do not include carbohydrate, free fatty acids and certain minor lipids, whereas CHN analysis quantifies essentially all organic components. Table 2 shows a summary of the SRI of total LDL and HDL as obtained by calibrated AnUC and by refractometry. The more accurate elemental mass was used for these results. The average SRI for LDL and HDL determined by AnUC was lower by 10% and 5% than that by refractometry. These unexpected results suggest the presence in both the LDL and HDL fractions of lipoprotein macromolecules close to the densities 1.061 ml/g and 1.200 g/ml. At these solution densities, given at 26 C, such molecules would not be expected to undergo significant flotation (or sedimentation) at 52,640 rpm and hence would not contribute to the flotation boundary and refractive index increment resolved by the schlieren optical system. This interpretation is supported by the presence in the second ml (LDL) and second ½ ml (HDL) of ca. 5% and 8% of lipoprotein macromolecules of density close to that in the preparative tube (see Table 1). The implications of this finding suggest the need for some increase in preparative fractionation time, possible revision of solvent densities used and analysis of both the top and infranatant fractions by other techniques.

In order to further characterize the significant subnatant macromolecular mass, samples $2VLH_1^0$ (total HDL) and $4VLH_{1.5}^0$ (HDL subnatant) from subject 7608 were dialyzed against a solution of d = 1.470 g/ml that contains NaBr in 0.194 molal NaCl. Flotation at 52,640 rpm for 64 min yielded the schlieren patterns shown in Figure 2. These results, and those from radial immunodiffusion, indicate the presence in the subnatant of 1.84 mg/ml albumin and 1.32 mg/ml HDL_3 lipoproteins. Because this fraction is at 4-fold concentration, the unrecovered HDL is 5.5% in the most extreme case. The much smaller HDL peak reflects the significantly reduced SRI of HDL in the 1.470 g/ml salt solution. For all 5 LDL and HDL samples, the CHN and compositional data indicate the unrecovered lipoproteins in the subnatants of a standard low and high density run (5) to be ca. 3%. Essentially no albumin was found in the LDL subnatant, but ca. 58% of the HDL subnatant mass was albumin.

TABLE 2

Evaluation of Lipoprotein Specific Refractive Increments (SRI) Δn/g/100 ml

	Total LDL		Total HDL	
Sample #	AnUC (n = 1)[d]	Refractometry[d]	AnUC[d]	Refractometry[d]
6569	0.001339	0.001754	0.001447 (n = 4)	0.001617
6661	0.001421	0.001579	0.001252 (n = 2)	0.001326
6662	0.001552	0.001502	0.001333 (n = 3)	0.001322
7608	0.001363	0.001500	0.001487 (n = 1)	0.001461
7684	0.001409	0.001528	0.001213 (n = 1)	0.001343
Mean SRI (5)	0.001417 (90.1%)[a]	0.001573	0.001347 (95.3%)	0.001414
Correction factor for old data[b]	1.087		1.106	
7072 (frozen 7 wk)[c]	0.001284	0.001446	0.001227 (n = 1)	0.001405
7609 (frozen 1 yr)[c]	0.001410	0.001591	0.001237 (n = 1)	0.001347
Mean SRI (2)	0.001347 (88.7%)	0.001519	0.001237 (89.9%)	0.001376

[a]Percent of lipoprotein refractive increment resolved by AnUC compared with refractometry.

[b]Old SRI values for total LDL = 0.00154 and for HDL = 0.00149 Δn/g/100 ml. New values include total mass evaluated by CHN elemental microanalysis and by refractive increment evaluated by AnUC schlieren Δn.

[c]Frozen sample at −70 C obtained earlier from subject 7608.

[d]All AnUC data and precision refractometry were performed at 26 C.

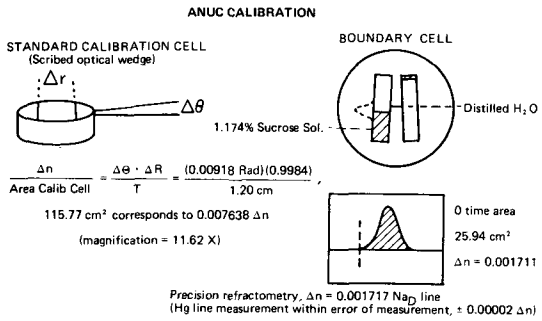

FIG. 2. Flotation in 1.470 g/ml of the total HDL fraction and subnatant from subject 7608. The subnatant HDL contamination is the slowly migrating HDL_3 when compared with the upper total HDL pattern.

In summary, the validated standard wedge calibration cell and the revised SRI for LDL and HDL significantly improve and confirm the accuracy of all AnUC runs. Thus, improved reliability and accuracy is possible for the AnUC as a reference standard for quantitative human lipoprotein analysis. Although the above results are in some ways unexpected, they provide a measure of absolute concentration correction to all previous and current AnUC data. Thus, total LDL and HDL concentrations using our previous SRI values of 0.00154 and 0.00149 Δn/g/100 ml should be corrected by factors of 1.087 and 1.106.

ACKNOWLEDGMENTS

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REFERENCES

1. Calibration Cell for the Model E Analytical Ultracentrifuge, Spinco Division, Beckman Instruments, Inc. Technical Bulletin E-TB-003C, February, 1963.
2. Armstrong, S.H., Budka, M.J.E., Morrison, K.C., and Hasson, M. (1947) J. Am. Chem. Soc. 69, 1747-1753.
3. Hanig, M., and Shainoff, J.R. (1956) J. Biol. Chem. 219, 479-486.
4. Lindgren, F.T., Nichols, A.V., Freeman, N.K., Wills, R.D., Wing, L., and Gullberg, J.E. (1964) J. Lipid Res. 5, 68-74.
5. Lindgren, F.T., Jensen, L.C., and Hatch, F.T. (1972) in Blood Lipids and Lipoproteins (Nelson, G.J., ed.) pp. 181-274, John Wiley-Interscience, NY.
6. Lindgren, F.T., Hulley, S.B., Adamson, G.L., Glines, L.A., and Kahlon, T.S. (1983) in Dietary Fats and Health (Perkins, E.G. and Visek, W.J., eds.) pp. 564-573, American Oil Chemists' Society, Champaign, IL.
7. Lindgren, F.T. (1975) in Analysis of Lipid and Lipoproteins (Perkins, E.G., ed.) pp. 564-573, American Oil Chemists' Society, Champaign, IL.
8. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
9. Shore, V.G., Butterfield, G., and Krauss, R.M. (1983) in Dietary Fats and Health (Perkins, E.G. and Visek, W.J., eds.) pp. 667-678, American Oil Chemists' Society, Champaign.

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Behavior of Sugar Derivatives in Procedures for Ganglioside Isolation

ALLAN J. YATES* and **JEAN K. WARNER**, *Division of Neuropathology, College of Medicine, Room 111 Upham Hall, Ohio State University, 473 W. 12th Avenue, Columbus, OH 43210*

ABSTRACT

A common method of studying ganglioside metabolism is to measure the amounts of radioactivity incorporated into ganglioside from a radiolabeled precursor. This requires that radioactive nonganglioside material be completely removed from the ganglioside fraction. Nucleotide sugars and aminosugars comprise an important source of such contaminants. Therefore, we have studied their behaviors in several procedures currently employed to isolate gangliosides. Over 50% of the radioactivity associated with several nucleotide sugars added to a brain homogenate is extracted with chloroform/methanol (2:1, v/v), and most of this is recovered in the upper phase of a Folch partition. Dialysis against water removes almost all of the free aminosugar but only 70% of nucleotide sugar. Treatment with alkaline phosphatase, phosphodiesterase and alkaline methanol followed by dialysis removes almost all of the nucleotide diphosphate sugars but only 88% of cytidine 5'-monophosphate sialic acid (CMP-NeuAc). Nucleotide sugars cannot be separated from gangliosides by Unisil or Iatrobead chromatography, but nucleotide diphosphate sugars and gangliosides are resolved with Sephadex LH-20 chromatography following treatment with phosphodiesterase and alkaline phosphatase. CMP-NeuAc was not satisfactorily separated from gangliosides using any of the procedures.
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INTRODUCTION

Gangliosides are sialic acid containing glycosphingolipids located mainly at the cell surface and found in virtually all vertebrate tissues studied. Their normal biological roles are unknown but researchers have considerable interest in them because of evidence suggesting that they may be involved in nerve regeneration (1), immunoregulation (2,3), receptor activities (4) and cellular growth control (5). In metabolic studies involving the radiolabeling of gangliosides by radioactive precursors, two major problems have arisen: (a) separation of labeled gangliosides from other radioactive metabolites (mainly aminosugars and nucleotide sugars); (b) preventing the loss of gangliosides, particularly during desalting procedures such as dialysis. Carter and Kanfer (6) studied the behavior of N-acetylgalactosamine, glucose, UDP-glucose and UDP-galactose in some of the procedures available at that time for ganglioside isolation. A few other reports on the behavior of such contaminants at some steps of some isolation procedures are scattered in the literature, usually as minor parts of major papers (7-10). However, no recent comprehensive study has been done on this subject. Because of the importance of such information in interpreting the data from experiments in which gangliosides are radiolabeled, we have studied the behavior of several nucleotide sugars and aminosugars during some of the critical procedures currently being used to isolate gangliosides. The results of that study form the basis of this report.

*To whom correspondence should be addressed.

MATERIALS AND METHODS

Materials

The following chemicals were purchased: uridine diphosphate [^{14}C-(U)] glucose, 327 mCi/mmol (UDP-glc); uridine diphosphate [^{14}C-(U)] galactose, 302 mCi/mmol (UDP-gal); uridine diphosphate N-acetyl [1-^{14}C]-D-glucosamine, 51.7 mCi/mmole (UDP-glcNac); uridine diphosphate N-acetyl [1-^{14}C]-D-galactosamine, 47.2 mCi/mmol (UDP-galNac); guanosine diphosphate [^{14}C(U)] fucose, 190 mCi/mmol (GDP-fuc); cytidine 5'-monophosphate [sialic-4-^{14}C] sialic acid, 1.6 mCi/mmol and 247 mCi/mmol (CMP-NeuAc); glucosamine hydrochloride, D-[1,6-^{3}H(N)], 32.5 Ci/mmol ($GlcNH_2$); Aquasol-2 Universal Liquid Scintillation Counting Cocktail from New England Nuclear, Boston, MA; DEAE Sephadex A-25 and Sephadex LH-20 from Pharmacia Fine Chemicals, Piscataway, NJ; Unisil (silicic acid) 200-325 mesh from Clarkson Chemical Co., Williamsport, PA; Iatrobeads from Iatron Labs, Tokyo, Japan; snake venom phosphodiesterase I and *E. Coli* alkaline phosphatase from Worthington Biochemicals Corps, Freehold, NJ; precoated thin layer plates (Silica Gel 60, 20 cm × 20 cm) from Merck, Darmstadt, Germany; microdialy-

sis tubes from Pope Scientific, Menominee, WI.

All chemicals and organic solvents were of reagent grade and solvents were redistilled before use. Purity of radiolabeled materials was determined by paper chromatography using S&S Orange Ribbon 589C paper (Schuleucher and Schuell, Inc., Keene, NH) using the following solvents: butanol/ethanol/water (50:32:18, v/v/v) for glucosamine hydrochloride; ethanol/1.0M ammonium acetate pH 3.8 (5:2, v/v) for UDP-glcNac; ethanol/1.0M ammonium acetate pH 5.0 (7:3, v/v) for all other nucleotide sugars. Purity for all of these was at least 94.0% and averaged 95.8%.

A mixture of gangliosides was isolated from normal human cerebral cortex (NHCG) according to the method of Suzuki (11).

Experimental Procedures

Extraction and partition. Aliquots of radiolabeled nucleotide sugars and $GlcNH_2$ (amounts in dpm given in Table 1) were added separately to homogenates of 1 g normal human cerebral cortex in 20 vol chloroform/methanol (2:1, v/v). Gangliosides were extracted and partitioned by the method of Suzuki (11) with 3 additional washes of theoretical upper phase without KCl. Aliquots were taken from the total unwashed lipid extract, upper phase and lower phase, and radioactivity in each was determined.

TABLE 1

Distribution of Nucleotide Sugars and Glucosamine Following Extraction and Folch Partition[a]

Radiolabeled compound	Initial amount	Total lipid extract	Upper phase	Lower phase
$GlcNH_2$	381	292	265	5.02
UDP-gal	102	39.4	47.1	0.18
UDP-galNac	93.2	70.1	74.5	0.20
CMP-NeuAc	98.0	66.5	69.9	0.12

[a]Values are dpm $\times 10^{-3}$.

Dialysis. In experiment I, samples of $GlcNH_2$ (235,000 dpm) were dissolved separately in 2 ml aliquots of water, transferred to mini dialysis bags and dialysed at 4 C against distilled water for several different periods of time with water changes every 2 hr. Contents of the dialysis bags were taken to dryness and radioactivity determined.

In experiment II, aliquots (0.2 nmol each; amounts in dpm given in Table 2) of nucleotide sugars were prepared for dialysis as above. Some contained NHCG equivalent to 100 mg cerebral cortex; others did not. Dialysis was for 48 hr at 4 C with 6 water changes.

In experiment III, aliquots of nucleotide sugars (0.2 nmol each; amounts in dpm given in Table 2) and NHCG as above were treated with phosphodiesterase and alkaline phosphatase (6) and dialysed as in experiment II. Identical samples received no enzyme treatment but were dialysed similarly.

In experiment IV, samples prepared identically to those in experiment III were subjected to alkaline methanolysis (12) by treating with 0.6N NaOH in methanol at room temperature and neutralizing with HCl, but did not receive the enzyme treatment. They were dialysed as in experiment II. Control samples were not methanolysed.

TABLE 2

Removal of Nucleotide Sugar Radioactivity by Dialysis[a]

Experiment[b]	UDP-glc	UDP-glcNac	UDP-gal	UDP-galNac	GDP-fuc	CMP-NeuAc
II A	–	–	122	20.9	–	94.6
B	–	–	26.3 (21.6)	1.23 (5.9)	–	15.9 (16.8)
C	–	–	42.6 (34.9)	20.8 (99.5)	–	33.5 (35.4)
III A	150	19.7	122	18.6	71.2	111
B	17.2 (11.5)	8.13 (41.3)	11.8 (9.7)	9.17 (49.3)	35.3 (49.6)	31.6 (28.5)
C	0.19 (0.1)	0.10 (0.5)	0.05 (0.04)	0.10 (0.5)	0.08 (0.1)	14.7 (13.2)
IV A	–	–	122	21.0	–	94.6
B	–	–	3.4 (2.8)	3.30 (15.7)	–	13.0 (13.7)

[a]Samples were dialysed for 48 hr against distilled water with 8 water changes at 6 hr intervals. Values refer to dpm $\times 10^{-3}$ radioactivity remaining within dialysis bag; number in brackets is percentage of original radioactivity remaining after dialysis.

[b]IIA, IIIA and IVA–Initial amounts of radioactivity before dialysis. Sample IIB was dialysed in absence of ganglioside; all others had human cerebral cortex gangliosides added before dialysis. IIIB received no enzyme treatment; IIIC was treated with alkaline phosphatase and phosphodiesterase before dialysis. IVB underwent alkaline methanolysis before dialysis.

Unisil columns. Known amounts of nucleotide sugars (ca. 100,000 dpm each) were loaded onto a 1 g Unisil column and sequentially eluted with chloroform (15 ml), chloroform/methanol (4:1, v/v, 15 ml), chloroform/methanol (1:1, v/v, 15 ml) and methanol (35 ml). UDP-Glc was also chromatographed on a Unisil column with gangliosides (100 μg NeuAc). Radioactivity in each 1 ml fraction was determined.

Iatrobead columns. Iatrobeads were prewashed according to the method of Momoi (13) and known amounts of nucleotide sugars (ca. 100,000 dpm each) were loaded onto separate 1 g columns in 5 ml chloroform/methanol (85:15, v/v). Columns were eluted with chloroform/methanol (85:15, v/v; 15 ml, including 5 ml to load) and chloroform/methanol (1:2, v/v, 40 ml).

DEAE Sephadex A-25 columns. These columns were prepared by first converting the resin to acetate by the method of Ledeen and Yu (14). Nucleotide sugars (100,000 dpm each) or $GlcNH_2$ (325,000 dpm) were added to separate dried aliquots of total lipid extract from 100 mg of normal human cerebral cortex. Each sample was then loaded onto a 1 g column in 50 ml chloroform/methanol/water (30:60:8, v/v/v) and eluted with 50 ml more of the same solvent (Fraction A). The columns were then eluted with 100 ml chloroform/methanol/0.8 M aqueous sodium acetate (30:60:8, v/v/v [Fraction B]). Because of low recoveries of radioactivity, in certain cases the columns were further eluted with 100 ml of chloroform/methanol/0.8 M aqueous KCl (30:60:2, v/v/v [Fraction C]). The fractions were collected and aliquots taken for counting in 10 ml Aquasol-2.

Sephadex LH-20 columns. Sephadex LH-20 was soaked in chloroform/methanol (1:1, v/v) overnight before use. Columns (17-18 mm o.d. × 370 mm) were prepared with 15 gm Sephadex LH-20 in the same solvent. Samples were loaded onto columns in 5 ml methanol and eluted isocratically with chloroform/methanol (1:1, v/v). Nucleotide sugars and aminosugars (100,000 dpm each) were chromatographed with and without NHCG (100 μg NeuAc). Other samples of nucleotide sugars (0.2 nmol each; amounts in dpm as in Table 2, experiment IIIA) with gangliosides (100 μg NeuAc) were subjected to phosphodiesterase--alkaline phosphatase treatment or alkaline methanolysis or both, and then passed through Sephadex LH-20 columns. For all of these columns, 1 ml fractions were collected by a Buchler Fractomette Alpha 400 into mini scintillation vials. The fractions were then dried under nitrogen and counted in 6 ml Aquasol-2. If gangliosides were present, 10% aliquots of the fractions were spotted on Merck TLC Silica Gel 60 plates and visualized by Svennerholm's (15) resorcinol spray, which detects 0.5 mg NeuAc.

Liquid scintillation counting. All radioactive samples were counted in Aquasol-2 using a Beckman LS-7500 Liquid Scintillation Counter. An external standard was used to correct for quenching and calculate dpm.

RESULTS

Initial Lipid Extraction and Folch Partition

Significant amounts of nucleotide sugars and $GlcNH_2$ are soluble in the solvents commonly used to extract gangliosides from wet tissue. Over 99% of the nucleotide hexoses and 98% of $GlcNH_2$ are in the upper phase following a Folch partition (Table 1).

Dialysis

Four sets of experiments were performed to determine the effects of several factors on the dialysis of $GlcNH_2$ and nucleotide sugars. Duration of dialysis was studied in the first set. After 12 hr of dialysis with 6 water changes, over 99.7% of free $GlcNH_2$ was removed both in the presence and absence of gangliosides. An additional 36 hr of dialysis with 14 more water changes only removed an additional 0.1% of the original 295 × 10^3 dpm $GlcNH_2$ (data not shown). The presence of gangliosides within the dialysis bag did not affect the dialysis of free $GlcNH_2$ but did retard the dialysis of nucleotide sugars (Table 2, experiment 2). In the third set of experiments, the effects of alkaline phosphatase and phosphodiesterase digestion of nucleotide sugars before dialysis were investigated. Dialysis of nonenzyme-treated samples in the presence of gangliosides removed between 54% and 91% of radioactivity. Dialysis following enzyme treatment removed almost all radioactive nucelotide diphosphate sugars but only 88% of CMP-NeuAc (Table 2, experiment 3). Dialysis following alkaline methanolysis removed a greater proportion of radioactivity than no treatment, but was not as effective as enzyme digestion (Table 2, experiment 4).

Unisil Column Chromatography

When chromatographed alone, GDP-fucose, UDP-galNac, UDP-glcNac and CMP-NeuAc eluted almost entirely in the methanol fraction (Fig. 1a). However, significant amounts of UDP-gal and UDP-glc eluted in the C/M (1:1, v/v) as well as in the methanol fraction. When chromatographed with gangliosides, UDP-

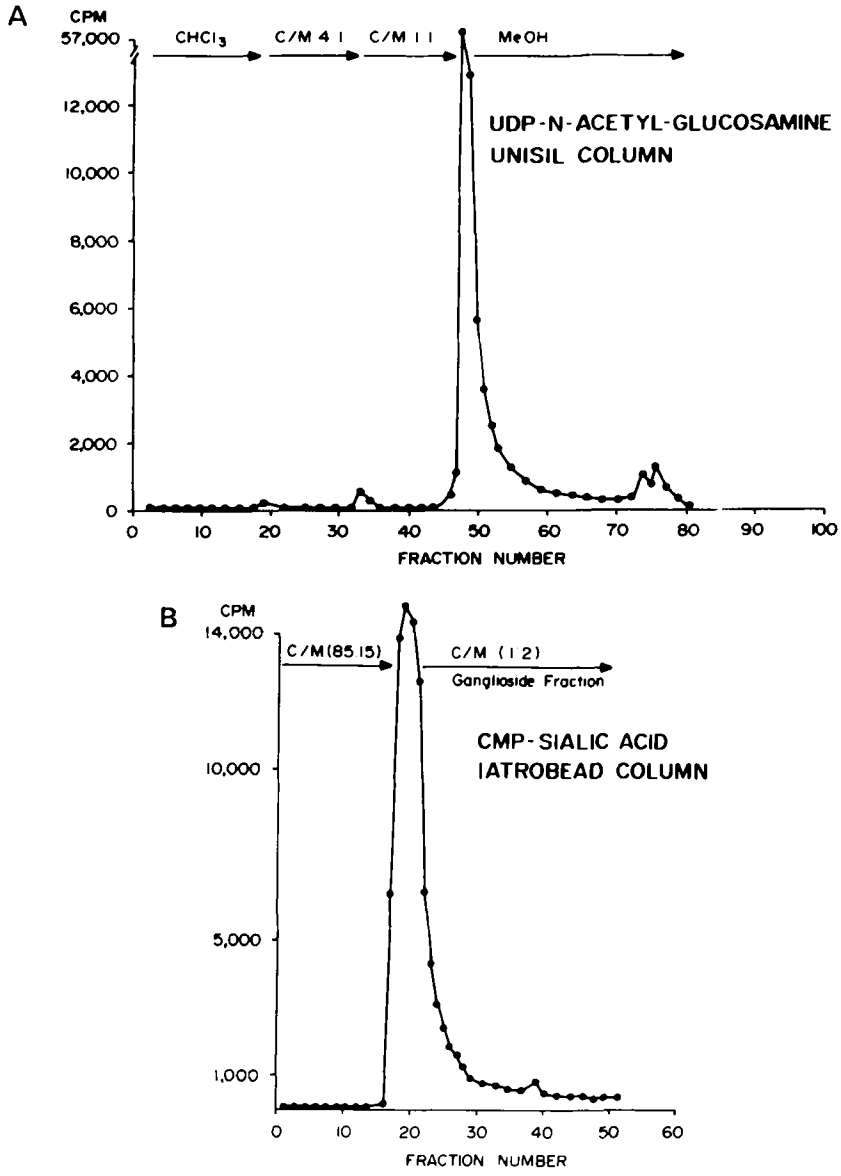


FIG. 1. Elution profiles of radioactivity from Unisil loaded with UDP-glcNac (a) and Iatrobead loaded with CMP-NeuAc (b).

glcNac eluted in the chloroform/methanol (1:1, v/v) and methanol fractions.

Iatrobead Column Chromatography

Six radiolabeled nucleotide sugars (UDP-gal, UDP-glc, UDP-galNac, UDP-glcNac, GDP-fucose and CMP-NeuAc) were passed through Iatrobead columns. Almost no radioactivity eluted in the chloroform/methanol (85:15, v/v) fraction. However, for all 6 compounds large amounts of radioactivity eluted as a peak as soon as the solvent was changed to chloroform/methanol (1:2, v/v) the fraction in which gangliosides elute (Fig. 1b).

DEAE Sephadex A-25 Column Chromatography

Six radiolabeled nucleotide sugars separately added to total lipid extract from 100 mg of normal human cerebral cortex were loaded onto DEAE Sephadex A-25 columns. Of the total amounts, between 1.3% and 7.7% was recovered in Fraction A (where neutral lipids

TABLE 3

Radioactivity Recovered from DEAE–Sephadex Column[a]

Fraction[b]	UDP-gal	UDP-Glc	UDP-galNac	UDP-glcNac	GDP-fucose	CMP-NeuAc	$GlcNH_2$
Initial	122	128	112	118	107	118	328
Fraction A	9.92	2.79	6.90	2.57	21.9	1.91	354
Fraction B	3.79	4.33	2.03	0.71	0.48	111	5.67
Fraction C	–	–	–	0.26	–	16.3	–

[a]Values represent dpm $\times 10^{-3}$ radioactivity.

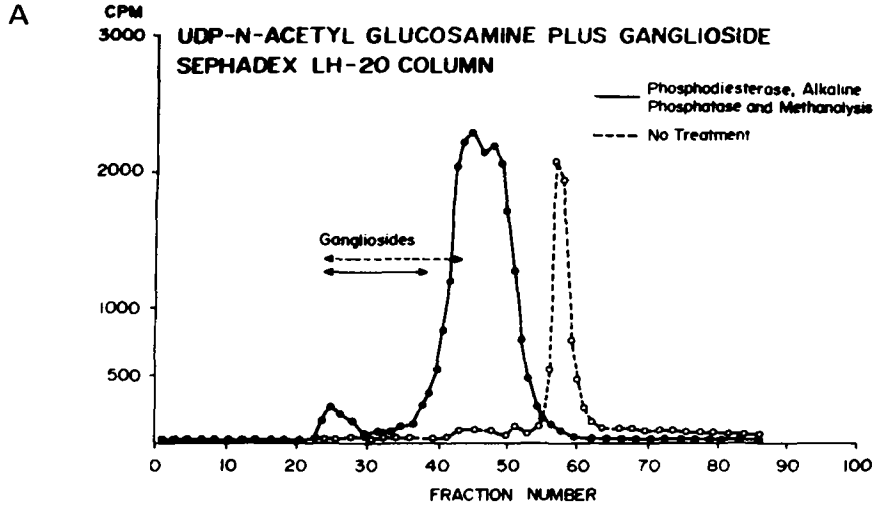
[b]Initial–the amount of radioactivity in the nucleotide sugar that was loaded onto the column in the presence of total lipids extracted from 100 mg normal human cerebral cortex. Fraction A–chloroform/methanol/water (30:60:8, v/v/v), 100 ml. Fraction B–chloroform/methanol/0.8M Na acetate in water (30:60:8, v/v/v), 100 ml. Fraction C–chloroform/methanol/0.8M KCl in water (30:60:2, v/v/v), 100 ml.

elute) except GDP-fucose, 20% of which eluted in the first fraction (Table 3). Only 0.4-3.1% of the total amount of these eluted with the acidic lipids (Fraction B) except CMP-NeuAc, 94.3% of which was recovered in Fraction B. Because of the low recoveries of these compounds (except CMP-NeuAc), an additional 100 ml chloroform/methanol/0.8M KCl (30:60:2, v/v/v) (Fraction III) was passed through 2 columns. Even this harsher solvent removed only another 0.2% UDP-GlcNac, but removed all of the remaining CMP-NeuAc. Almost all of the radiolabel from $GlcNH_2$ was recovered in Fraction A (Table 3).

Sephadex LH-20 Column Chromatography

Normal human cerebral cortex gangliosides always eluted in fractions 20-43. When chromatographed alone, most of the radioactivity associated with the nucleotide sugars we studied (except GDP-fucose) was recovered from Sephadex LH-20 columns within these same fractions. GDP-fucose eluted as a large single peak between fractions 40 and 50. However, when NHCG was chromatographed at the same time as either UDP-galNac or UDP-glcNac the retention times of the nucleotide sugars were increased so that only a very small proportion of either of them co-eluted with the gangliosides (Fig. 2a). A similar elution pattern was seen when $GlcNH_2$ was chromatographed with NHCG. However, CMP-NeuAc eluted both with and, later than NHCG.

When UDP-glcNac was subjected to alkaline methanolysis before loading it onto a column, a significant amount of radioactivity usually co-eluted with the NHCG (Fig. 2b). However, treatment of both UDP-glcNac and UDP-galNac (Fig. 2c) with phosphodiesterase and alkaline phosphatase resulted in the radioactivity eluting later than NHCG. Treatment of UDP-glcNac with both enzymes and alkaline methanolysis resulted in the separation of most of the radioactivity from NHCG (Fig. 2a). CMP-NeuAc could not be separated from NHCG by treating it with these 2 enzymes (Fig. 2c).



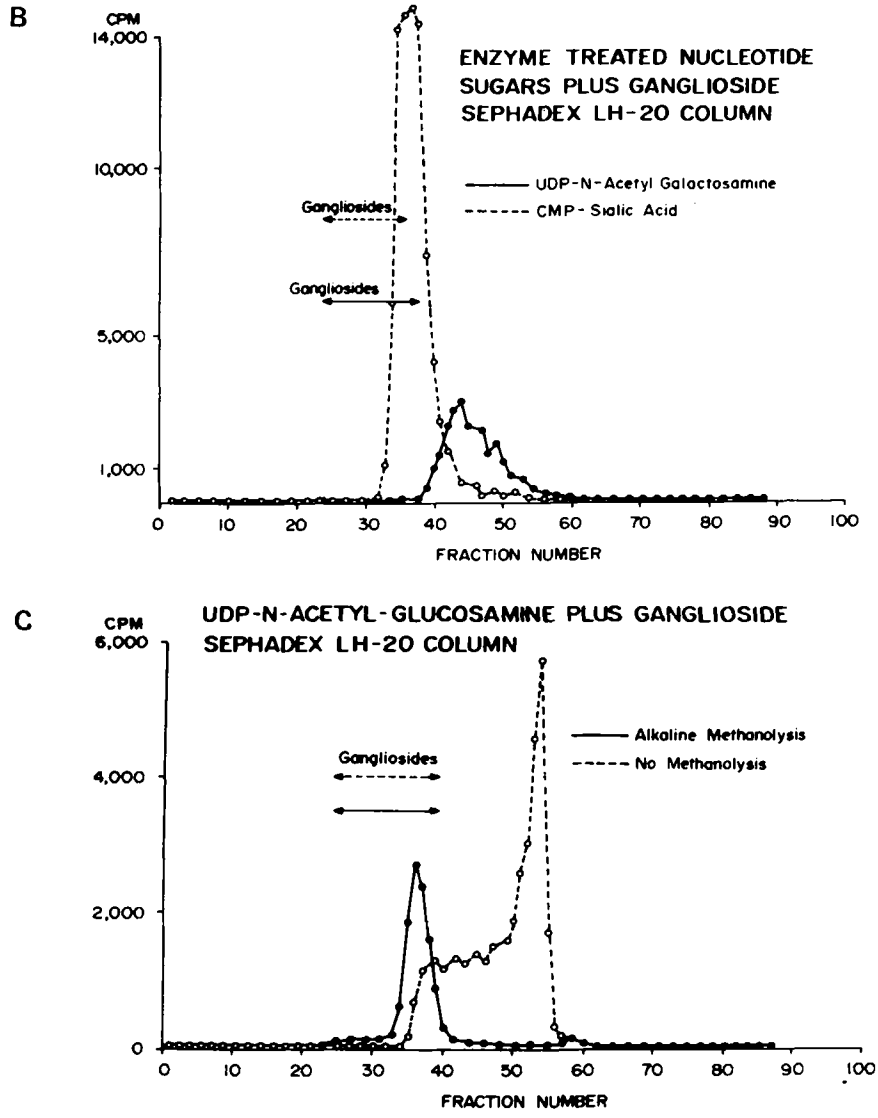


FIG. 2. Elution profiles of radioactivity from Sephadex LH-20 columns loaded with: (a) gangliosides and UDP glcNac or ganglioside and UDP-glcNac treated with phosphodiesterase, alkaline phosphatase and alkaline methanolysis; (b) gangliosides and either UDP-glcNac or UDP-glcNac subjected to alkaline methanolysis; (c) gangliosides and either UDP-galNac or CMP-NeuAc treated with both phosphodiesterase and alkaline phosphatase.

DISCUSSION

Two physical properties of gangliosides, which have been exploited in some isolation procedures, are their amphiphilic nature and ability to form micelles in water. However, these properties are also probably responsible for the difficulty in removing some contaminants, e.g., free aminosugars and nucleotide sugars, from ganglioside preparations. Chloroform/methanol (2:1, v/v) can dissolve relatively large amounts of $GlcNH_2$ and nucleotide sugars in tissue homogenates, and virtually all of these substances present in the total lipid extract of tissues goes into the upper phase of the Folch partition. Therefore, determinations of radio-

activity in chloroform-methanol extracts or Folch upper phases cannot be equated with the amount of ganglioside radioactivity in extracted tissue if they contain any radioactive nucleotide sugar or $GlcNH_2$. This is probably also true for other aminosugars but we did not study them.

Dialysis of gangliosides against water has been a common method of removing low molecular weight contaminants from gangliosides. The critical micelle concentration of gangliosides is still a matter of controversy but is not higher than 10^{-4} M and probably closer to 10^{-8} M (16). Minimal amounts are lost when gangliosides are present as micelles in water during dialysis (17,18). Our results indicate that within 12 hr almost all of the $GlcNH_2$ can be removed from ganglioside samples by dialysis with frequent water changes. This is not the case with nucleotide sugars, significant amounts of which still remain in the ganglioside fraction after 48 hr of dialysis with several water changes. This is probably caused by the trapping of the nucleotide sugars in the ganglioside micelles possibly on the basis of hydrophobic interactions. Gangliosides can form such mixed micelles (16), and Kanfer reported this phenomenon for UDP-gal and UDP-glc (6,7). He recommended treating samples with phosphodiesterase and alkaline phosphatase before dialysis to split the nucleotide sugars into readily dialysable fragments. Our results confirm that this treatment, plus dialysis, effectively removes nucleotide diphosphate sugars. However, ca. 10% CMP-NeuAc radioactivity remains with the ganglioside fraction. The reasons for this are not entirely clear, but although CMP-NeuAc is resistant to phosphodiesterase treatment, it may undergo nonenzymatic hydrolysis to CMP and NeuAc during dialysis. CMP-NeuAc is relatively unstable. Alkaline methanolysis, followed by dialysis, was not as effective as the enzyme treatment in removing nucleotide sugars. Therefore, if a procedure calls for alkaline methanolysis, usually to remove phospholipids (12), we suggest that the methanolysis step should follow enzyme digestion.

Unisil and Iatrobead column chromatography are commonly used as a final step in ganglioside purification (14,19), usually to remove lipid contaminants less polar than gangliosides. Our results show that these columns do not effectively separate gangliosides and nucleotide sugars when eluted with solvents usually employed. SilicAR also fails to separate these compounds (6).

DEAE Sephadex A-25 column chromatography is used in ganglioside isolation to separate neutral and Zwitterionic lipids from acidic lipids. Over 90% of CMP-NeuAc radioactivity loaded onto the column is recovered in the acidic lipid fraction with gangliosides. The recovery of the other 5 nucleotide sugars we studied was quite low. This is probably because each has 2 phosphate groups that would interact electrostatically with the solid phase to a greater extent than CMP-NeuAc. Additional evidence for this is the finding of Ueno et al. (8) that gangliosides can be separated from UDP-gal on Sephadex G-50. In our study, the small amount of the diphosphonucleotide sugars that did elute from the DEAE-Sephadex column probably were electrically neutralized by a positive counterion. Virtually all of the free $GlcNH_2$ was recovered in the neutral lipid fraction, as would be expected. Therefore, DEAE-Sephadex column chromatography removes most of the potentially contaminating free and nucleotide sugars from gangliosides, with the exception of CMP-NeuAc. This is in striking contrast to results obtained with Sephadex G-50, with which a complete separation of nucleotide sugars and gangliosides can be obtained with quantitative recoveries of nucleotide sugars (9).

The main purpose for using a Sephadex LH-20 column during ganglioside isolation is to remove inorganic salts. Theoretically, it might also remove other low molecular weight substances, e.g. free aminosugars and nucleotide sugars. Almost all of the free $GlcNH_2$ elutes after the gangliosides. With exception of GDP-fucose, all the nucleotide sugars we studied, when run in the absence of gangliosides, eluted in the same fractions in which gangliosides consistently elute. However, the elution profiles of UDP-glcNac, UDP-galNac and CMP-NeuAc were altered when chromatographed with gangliosides. The first two were retarded and the bulk of each eluted later than gangliosides. CMP-NeuAc eluted both with and later than the gangliosides. The reasons for these changes in mobility in the presence of gangliosides are unknown.

Alkaline methanolysis has been included as a step in the isolation of gangliosides to remove glycerophospholipids (12,14). Such a treatment of a mixture of UDP-glcNac and gangliosides resulted in slightly variable elution profiles of the radioactivity. Sometimes it eluted with and sometimes later than the gangliosides. This is probably a consequence of the multiple products formed. Most of the ^{14}C-labeled products that elute are probably GlcNac-1-phosphate and GlcNac-2-phosphate (20). The reason that most of these radiolabeled reaction products elute sooner than their parent nucleotide sugars is that the retarding aromatic interactions of the

nonradioactive purines and pyrimidines (which are retarded because of their aromatic interactions with the stationary phase) have been dissociated from the radiolabeled $GlcNH_2$. A similar effect has been noted when UDP-glcNac was treated with phosphodiesterase (results not shown) and the same explanation probably applies in this case as with the alkaline methanolysis. Treating UDP-glcNac with combined phosphodiesterase and alkaline phosphatase resulted in most of the radioactivity eluting later than the gangliosides, similar to free $GlcNH_2$. Indeed, the products of phosphodiesterase treatment would be expected to be dephosphorylated by alkaline phosphatase yielding free $GlcNH_2$. Therefore, effective removal of radiolabeled free aminosugars and nucleotide diphosphate sugars can be obtained by digesting them with these 2 enzymes before Sephadex LH-20 column chromatography. However, because sialic acid does not have a hydroxyl group on carbon-2, CMP-NeuAc is not split by phosphodiesterase and significant amounts of radioactivity co-elute with gangliosides. If separating CMP-NeuAc from gangliosides is imperative, the method of Yohe et al. (9) can be employed.

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REFERENCES

1. Gorio, A., and Rapport, M. (1981) Gangliosides in Neurological and Neuromuscular Function and Repair, Raven Press, NY.
2. Yates, A.J., Hitchcock, C.L., Stewart, S.S., and Whisler, R.L. (1980) ACS Symposium Series 128, 419-433.
3. Whisler, R.L., and Yates, A.J. (1980) J. Immunology 125, 2106-2111.
4. Fishman, P.H., and Brady, R.O. (1976) Science 194, 906-914.
5. Icard-Liepkalns, C., Liepkalns, V.A., Yates, A.J. Rodriguez, Z.R., and Stephens, R.E. (1982) J. Cell Physiol., 186-191.
6. Carter, T.P., and Kanfer, J. (1973) Lipids 8, 537-548.
7. Kanfer, J. (1969) in Methods in Enzymology (Lowestein, J.M., ed.), pp. 660-664, Academic Press, NY.
8. Ueno, K., Ando, S., and Yu, R.K. (1978) J. Lipid Res. 19, 813-821.
9. Yohe, H.C., Ueno, K., Chang, N.-C., Glaser, G.H., and Yu, R.K. (1980) J. Neurochem. 34, 560-568.
10. Wells, M.A., and Dittmer, J.C. (1963) Biochem. 2, 1259-1263.
11. Suzuki, K. (1965) J. Neurochem. 12, 629-638.
12. Dawson, G. (1972) J. Lipid Res. 13, 207-219.
13. Momoi, T., Ando, S., and Nagai, Y. (1976) Biochim. Biophys. Acta 441, 488-497.
14. Ledeen, R.W., and Yu, R.K. (1982) in Methods in Enzymology 83, 139-191.
15. Svennerholm, L. (1957) Biochim. Biophys. Acta 24, 604-611.
16. Corti, M., Degiorgio, V., Ghidoni, R., and Sonnino, S. (1982) in Solution Behavior of Surfactants (Mittal, K.L. and Fendler, E.J., eds.), Vol. 1, pp. 573-594, Plenum Publishing, NY.
17. Kanfer, J., and Spielvogel, C. (1973) J. Neurochem. 20, 1483-1485.
18. Ghidoni, R., Sonnino, S., and Tettamanti, G. (1978) Lipids 13, 820-822.
19. Yates, A.J., and Wherret, J.R. (1974) J. Neurochem. 23, 933-1003.
20. Paladini, A.C., and Leloir, L.F. (1952) J. Biol. Chem. 51, 426-430.

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COMMUNICATIONS

The Biosynthesis of n-[$^2H_7$] Fatty Acids by *Arthrobacter globiformis* from [U-$^2H_{15}$] Octanoic Acid

ROBERT H. WHITE, *Department of Biochemistry and Nutrition, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061*

ABSTRACT

The principal fatty acids present when *Arthrobacter globiformis* is grown on a glycine medium free of normal fatty acids were found to be the C15 and C16 anteiso fatty acids; only a small amount of the normal fatty acids (C14 and C16) were present. Cells grown on the same medium but supplemented mented with 0.1 mg/ml [U-$^2H_{15}$] octanoic acid were found to contain an increased amount of the normal fatty acids and these fatty acids were found to be labeled with 7 deuteriums. I concluded that the octanoic acid is degraded by β-oxidation in these cells to [U-$^2H_7$] butyryl-CoA, which then competes with 2-methylbutyryl-CoA for the initiation of fatty acid biosynthesis.
Lipids 19:570-572, 1984.

INTRODUCTION

Methyl-branched iso and anteiso long-chain fatty acids are widely distributed in bacterial lipids, where they normally occur along with a small amount of normal fatty acids (1). Both types of fatty acids are biosynthesized by the repeated condensation of malonyl-CoA with a starter acyl-CoA group. The biosynthetic difference between the branched- and straight-chain fatty acids lies in the starter acyl-CoA group used to initiate the biosynthesis (2,3). For the branched-chain fatty acids, the metabolic products of the branched-chain amino acids supply the required precursors. For the terminally branched iso fatty acids with an even number of carbons, the required initiator, isobutyryl-CoA, originates from valine via α-ketoisovalerate. Similarly, the terminally branched iso acids with a odd number of carbons come from leucine and the subterminally branched anteiso acids come from isoleucine (4,5). The assumption is generally made that the normal odd- and even-numbered fatty acids are initiated from either acetyl-CoA or propionyl-CoA, respectively (6).

In their work on fatty acid biosynthesis in *Bacillus subtilis* and *Escherichia coli*, Butterworth and Bloch (7) demonstrated that one of the major factors governing the final pattern of fatty acids produced by these organisms was determined by the specificity of the initial acyl-CoA–acyl carrier protein transacylase. In *B. subtilis* this enzyme mainly produces branched-chain acids; it has a high degree of preference for the branched-chain primers and virtually excludes initiation of straight-chain fatty acid synthesis by acetyl-CoA. In order to determine how cells that produce mostly branched-chain fatty acids biosynthesize n-fatty acids this investigator conducted an in vivo stable isotope experiment using *Arthrobacter globiformis*. This work demonstrated that the n-fatty acids produced by this bacteria are initiated exclusively from butyryl-CoA, not acetyl-CoA.

MATERIALS AND METHODS

A. globiformis (ATCC 8010) was grown at 28 C in 450 ml of the medium described by Kochi and Kikuchi (8) in 2.8 l Fernback flasks, which were rotated at 100 RPM unless otherwise indicated. Cells were maintained on slants of the same medium and transferred to the growth medium after 24 hr growth at 28 C. After growth at 28 C for 18 hr, the cells were removed by centrifugation and the entire cell pellet hydrolyzed with acid (9). The fatty acids were extracted from the acid-hydrolyzed cells with methylene chloride and converted into the methyl esters with diazomethane. Gas chromatography (GC) and GC-MS (mass spectrometry) of the resulting methyl esters were used to establish the identity of the fatty acids. The methyl esters were separated in each case on a 1/8 in. × 6 ft glass column packed with 10% SP-2100 on Chromosorb Q, with the temperature programmed from 150 C to 250 C at 10 C/min. Quantitation of the fatty acids was based on peak areas and is corrected for the relative molar response of each acid (10).

[U-$^2H_{15}$] Octanoic acid (98 atom % ^2H) was obtained from Merck Sharp and Dohme of Canada and was added to the growth medium as indicated.

TABLE 1

Fatty Acids of *Arthrobacter globiformis* Grown on a Glycine Medium in the Presence and in the Absence of 0.1 mg/ml [U-$^2H_{15}$]Octanoic Acid

Fatty acid	Without octanoic acid	With octanoic acid
Anteiso C14	3.4[a]	1.4
n-C14	0.9	3.9 (76.9% d_7)
Anteiso br-C15	76.9	64.4
Anteiso br-C16	9.9	5.3
n-C16	1.6	16.8 (74.0% d_7)
Anteiso br-C17	7.2	6.7

[a]Data expressed as the percentage of the total fatty acids.

RESULTS AND DISCUSSION

Analyses, as their methyl esters, of the fatty acids isolated from *A. globiformis* grown in the absence of octanoic acid showed the major fatty acids to be branched-chain acids (Table 1). These acids were identified as antieso acids by their GC retention times, which were shorter than those for the normal fatty acids of the same molecular weight (11), and by their mass spectra, i.e., each branched acid had a m/z (M^+-29) ion that was more intense than the m/z (M^+ -31) ion (12). In addition, the anteiso fatty acids have been characterized as the major fatty acids in other species of *Arthrobacter* (13). The cells also contained a small percentage of the normal C14 and C16 fatty acids, which were characterized by comparing their GC retention times and mass spectrum with that of knowns.

Growth of the cells in the presence of octanoic acid, however, resulted in a marked increase in the abundance of the normal C14 and C16 fatty acids (Table 1). This could indicate either that the octanoic acid chains were extended to the C14 and C16 acids or that it underwent a β-oxidation to a smaller fragment, which then initiated the biosynthesis of the nonbranched fatty acids. That only the latter occurred became clear because greater than 75% of the normal fatty acids isolated from the cells grown with the [U-$^2H_{15}$] octanoic acid contained 7 deuterium. Thus, the octanoic acid was being degraded by β-oxidation to [U-$^2H_7$]butyric-CoA, which had, in turn, been extended to the C14 and C16 n-fatty acids. The metabolism of octanoic acid in these cells by β-oxidation is consistent with the known ability of *Arthrobacter* species to grow aerobically with fatty acids as their sole carbon source (14) and contrasts sharply with fatty acid metabolism in *E. coli*, which is unable to metabolize fatty acids smaller than C14 because of the inability of these fatty acids to induce the enzymes of fatty acid oxidation (15,16). Thus, the synthesis of the n-fatty acids presumably occurred in *A. globiformis* by the substitution of butyryl-CoA for the α-methylbutyryl-CoA, the starter acyl-CoA expected to initiate the synthesis of the anteiso acids present in these cells (6). Had the octanoic acid been degraded completely to acetyl-CoA, and had this acetyl-CoA then served to initiate n-fatty acid biosynthesis, then some of the n-C14 and n-C16 fatty acids would be expected to contain 3 deuteriums. Because this was not observed, the butyrate must be assumed to serve to initiate all n-fatty acid synthesis in these cells. That portion of n-fatty acids not containing deuterium can be explained by fatty acid biosynthesis from unlabeled butyryl-CoA produced by the cells.

This observation was indirectly observed by Kaneda in his work on the biosynthesis of fatty acids in *B. subtilis* (3). Kaneda observed that label from both pyruvate-2-^{14}C and pyruvate-3-^{14}C was incorporated to a greater extent into the methylene carbons of palmitic acid than into the 2 terminal carbons (C16 and C15). This indicated that acetyl-CoA was not the acyl-CoA responsible for the biosynthesis of the palmitic acid present in these cells. Because butyric acid increased the production of n-fatty acids, Kaneda suggested (3), as is reported here, that butyryl-CoA serves as the initiator of fatty acid biosynthesis in bacteria. These observations explain how n-fatty acids can be produced in cells such as *B. subtilis*, which contain an acyl-CoA–acyl carrier protein transacylase this is not able to initiate fatty acid biosynthesis with acetyl-CoA (7). Only butyryl-CoA initiates n-fatty acid biosynthesis in cells that contain mostly branched-chain acids.

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REFERENCES

1. Polgar, N. (1971) Topics in Lipid Chemistry 2, 207-246.
2. Horning, M.G., Martin, D.B., Karmen, A., and Vagelos, P.R. (1961) J. Biol. Chem. 236, 669-672.
3. Kaneda, T. (1963) J. Biol. Chem. 238, 1229-1235.
4. Lennarz, W.J. (1961) Biochem. Biophys. Res. Commun. 6, 112-116.

5. Wegner, G.H., and Foster, E.M. (1963) J. Bacteriol. 85, 53-61.
6. Bressler, R., and Wakil, S.J. (1961) J. Biol. Chem. 236, 1643-1651.
7. Butterworth, P.H.W., and Bloch, K. (1970) Eur. J. Biochem. 12, 496-501.
8. Kochi, H., and Kikuchi, G. (1969) Arch. Biochem. Biophys. 132, 359-369.
9. White, R. (1980) Biochemistry 19, 9-15.
10. Wilson, R.H., Doty, V.E., Rencz, K.H., and Schram, A.C. (1966) J. Lab. & Clin. Med. 67, 87-97.
11. Horning, E.C., Karmen, A., and Sweeley, C.C. (1964) Progr. Chem. Fats and Other Lipids 7, 167-246.
12. Ryhage, R., and Stenhagen, E. (1960) J. Lipid Res. 1, 361-390.
13. Nesterenko, O.A., Andreev, L.V., Nogina, T.M., and Shkarula, V.V. (1980) Mikrobiol. Zh. (Kiev) 42, 288-293.
14. Buchanan, R.E., and Gibbons, N.E. (1974) Determinative Bacteriology, pp. 618-622, Williams & Wilkins Company, Baltimore, MD.
15. Weeks, G., Shapiro, M., Burns, R.O., and Wakil, S.J. (1969) J. Bacteriol. 97, 827-836.
16. Overath, P., Pauli, G., and Schairer, H.U. (1969) Eur. J. Biochem. 7, 559-574.

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Incorporation of Arachidonic Acid and Eicosapentaenoic Acid into Phospholipids by Polymorphonuclear Leukocytes in vitro

JEN-SIE TOU, *Department of Biochemistry, Tulane University School of Medicine, New Orleans, LA 70112*

ABSTRACT

The present study demonstrated that the patterns of the incorporation of [1-^{14}C] arachidonic acid and [1-^{14}C]eicosapentaenoic acid into individual phospholipids by polymorphonuclear leukocytes were similar. However, human leukocytes exhibited higher activity than guinea pig peritoneal leukocytes in the formation of arachidonoyl- and eicosapentaenoyl-phosphatidic acid. Cells from both origins showed a decrease of label in phosphatidylcholine accompanied by an increase of label in phosphatidylethanolamine after a longer period (30-120 min) of incubation, suggesting that part of the arachidonoyl or eicosapentaenoyl moiety in phosphatidylethanolamine may be derived from that of phosphatidylcholine. The observed difference between human cells and elicited cells in the time-course of the incorporation of both fatty acids into phosphatidylcholine and phosphatidylethanolamine appears to be due to different contents of the diacyl and ether-linked class compositions of these phospholipids in cells from different origins. Both labeled fatty acids were incorporated more rapidly into the diacyl-linked class, but were retained to a greater extent in alkylacyl-phosphatidylcholine and alkenylacyl-phosphatidylethanolamine. The data suggest that, in addition to alkylacyl-phosphatidylcholine and phosphatidylinositol, alkenylacyl-phosphatidylethanolamine may be an important endogenous source of arachidonic acid and eicosapentaenoic acid in stimulated human leukocytes.

Lipids 19:573-577, 1984.

Eicosapentaenoic acid, 20:5(n-3), is a major unsaturated component of fish lipid. It has been shown to be a little more active than arachidonic acid, 20:4(n-6), toward 5-lipoxygenase in guinea pig peritoneal polymorphonuclear leukocytes (PMNS) (1). It is metabolized to 5,12-dihydroxyeicosatetraenoic acid or leukotriene B_5 (LTB_5) by these cells in response to ionophore A23187, and LTB_5 exhibits 10- to 30-fold less chemotactic potency for human PMNS than 5,12-dihydroxyeicosatetraenoic acid or leukotriene B_4 (LTB_4) (2). However, our knowledge is lacking about the esterification of exogenous eicosapentaenoic acid to phospholipids by PMNS. The present study was undertaken to compare the incorporation of exogenous [1-^{14}C]20:4(n-6) into phospholipids with that of [1-^{14}C]20:5(n-3) by intact PMNS in vitro. Although studies on the metabolism of arachidonic acid by PMNS in vitro have been carried out with cells either from human blood or from animal peritoneal exudates, elicited PMNS differ from human blood PMNS in a number of respects (3,4). In the present study, PMNS from human blood and from guinea pig peritoneal exudates were compared in their incorporation of radiolabeled arachidonic acid [20:4(n-6)] and eicosapentaenoic acid [20:5(n-3)] into phospholipids.

MATERIALS AND METHODS

Preparation of PMNS

Human venous blood was collected from normal donors using heparin (1000 Units/50 ml blood) as an anticoagulant. The heparinized blood was diluted with an equal volume of Hanks balanced salt solution. In a 50 ml centrifuge tube the diluted heparinized blood was layered over one-third volume of Lymphocyte Separation Medium (Bionetics). After centrifuging the tubes at 400 x g for 30 min, PMNS and erythrocytes were found in the lower cell layer. After sedimentation of erythrocytes with two volumes of 3% dextran T500 (Pharmacia) and hypotonic lysis of contaminating erythrocytes, PMNS were washed and resuspended in Krebs-Ringer phosphate buffer modified to contain 1.3 mM $CaCl_2$ and 5 mM glucose at a concentration of 20×10^6 cells/ml. Cell counts were made in a hemocytometer, and cell viability was measured by trypan blue exclusion test. Cell preparations contained more than 95% PMNS. Guinea pig peritoneal PMNS were prepared as described previously (5). PMNS were collected 14 hrs after intraperitoneal injection of 12% sodium caseinate solution to guinea pigs.

Incubation of Cells with Radiolabeled Fatty Acid

[1-^{14}C]Arachidonic acid (52.9 Ci/mol, New England Nuclear Corp.) or [1-^{14}C]eicosapentaenoic acid (55.5 Ci/mol, New England Nuclear Corp.) was dissolved in dimethylsulfoxide (DMSO) and mixed with fatty acid-free bovine serum albumin (Mile Laboratory, 4 mg/ml 0.9% NaCl). The tubes, each containing 2.54 x 10^5 dpm of [1-^{14}C]arachidonic acid or 2.37 x 10^5 dpm of [1-^{14}C]eicosapentaenoic acid, were preincubated at 37 C for 30 min and then mixed with 20 x 10^6 PMNS from human blood or from guinea pig peritoneal exudates in a final volume of 2 ml and further incubated. The final concentration of bovine serum albumin in the incubation medium was 0.1 mg/ml. The DMSO, whose final concentration in the incubation medium was 0.025%, had no adverse effect on cell viability. Incubations were performed at 37° and stopped by the addition of 10 ml methanol to each tube.

Lipid Extraction and Analysis

Lipids were extracted according to Bligh and Dyer (6). Phospholipids were resolved by two-dimensional thin-layer chromatography on silica gel H (Analtech) and analyzed as described previously (7). The chromatogram was developed with chloroform-methanol-28% aqueous ammonia (65:25:5, v/v) in the first dimension and then with chloroform-methanol-acetic acid-water (85:10:40:4.5, v/v) in the second dimension.

PC and PE purified according to (8) were each treated with *Bacillus cereus* phospholipase C (Sigma) and followed by acetylation of the diglycerides (9). The resultant 1-radyl-2-acyl-3-acetylglycerols were resolved into 1-alkenyl-2-acyl-3-acetylglycerol, 1-alkyl-2-acyl-3-acetylglycerol and 1,2-diacyl-3-acetylglycerol by thin-layer chromatography (TLC) according to (10) on silica gel H (Analtech). The radioactivity of each lipid class was measured by liquid scintillation.

RESULTS AND DISCUSSION

The present study demonstrated that the patterns of the incorporation of 20:4(n-6) and 20:5(n-3) into individual phospholipids by PMNS were similar. It is likely that 20:4(n-6) in PMNS will be replaced by 20:5(n-3) when animals are fed a diet containing fish lipid. The lower chemotactic potency of LTB_5 derived from 20:5(n-3) than that of LTB_4 derived from 20:4(n-6) (2) will consequently alleviate the process of inflammation.

Figure 1 shows the similarity of the time-course of the incorporation of [1-^{14}C]20:4(n-6) and [1-^{14}C]20:5(n-3) into phosphatidic acid (PA) by PMNS from human blood and from guinea pig peritoneal exudates. However, human PMNS exhibited higher activity than guinea pig peritoneal PMNS in the formation of [1-^{14}C]20:4(n-6)- and [1-^{14}C]20:5(n-3)-PA. The labeled PA may participate in the biosynthesis of other phospholipids and neutral glycerolipids.

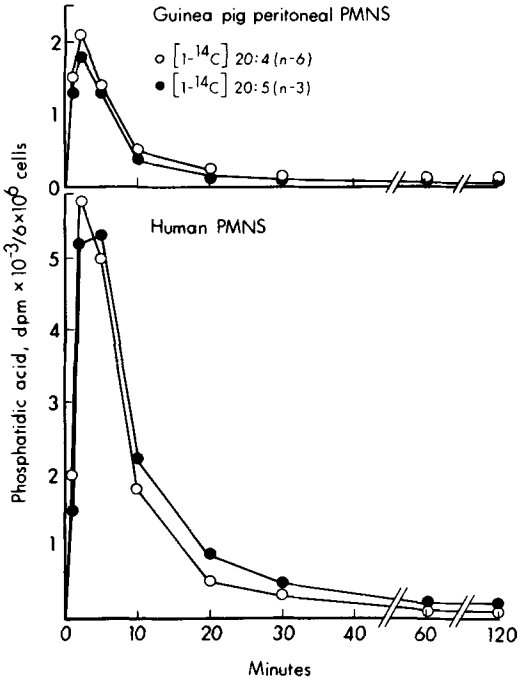

FIG. 1. The incorporation of [1-^{14}C]20:4(n-6) and [1-^{14}C]20:5(n-3) into PA by PMNS as a function of time. PMNS (20 x 10^6 cells) from human blood or from guinea pig peritoneal exudates were incubated at 37 C at the indicated time with [1-^{14}C]20:4(n-6) (2.54 x 10^5 dpm) or [1-^{14}C]20:5(n-3) (2.37 x 10^5 dpm) as described in Materials and Methods. Each point represents the average value from three experiments.

The labeling patterns of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) by both fatty acids in human PMNS were different from those in guinea pig peritoneal PMNS. In human PMNS the radioactivity of PC reached its maximum after 30 min incubation and started to decline, whereas that of PE and phosphatidylinositol (PI) continued to increase (Fig. 2). In guinea pig peritoneal PMNS (Fig. 3), the radioactivity of PC reached its maximum between 20 and 30 min; at 120 min it was approximately 60% of that at 20 min. During the incubation period the phosphorus content of each phospholipid remained unchanged. The observed decline in the radioactivity of PC appears to be the consequence of an exhaustion

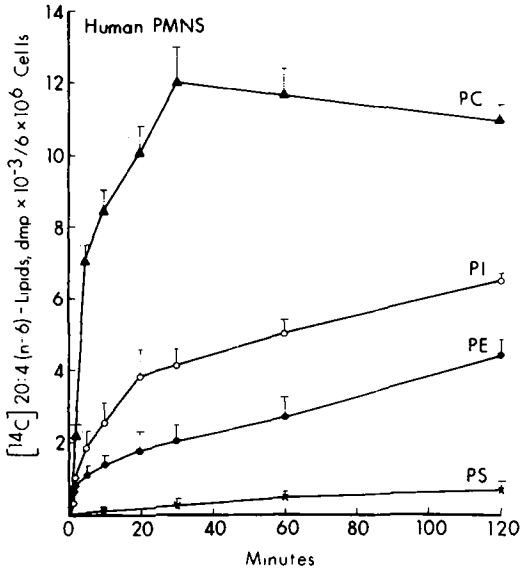

FIG. 2. The incorporation of [1-^{14}C]20:4(n-6) into PC, PE and PI by human PMNS as a function of time. Incubation conditions and phospholipid analysis were described in Materials and Methods. Each point represents the average value ± S.D. from three experiments.

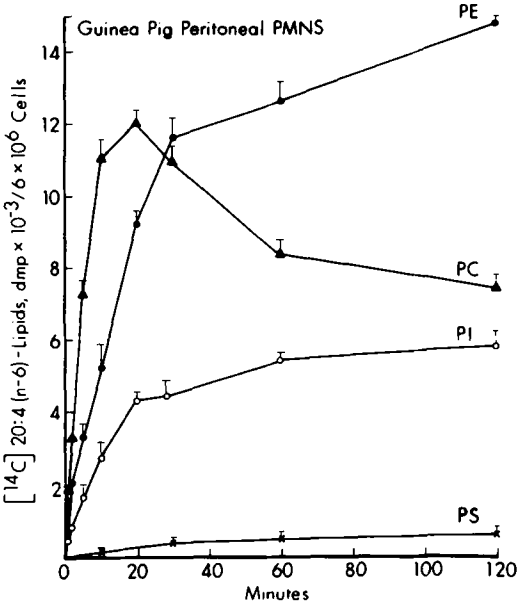

FIG. 3. The incorporation of [1-^{14}C]20:4(n-6) into PC, PE and PI by guinea pig peritoneal PMNS as a function of time. Incubation conditions and phospholipid analysis were described in Materials and Methods. Each point represents the average value ± S.D. from three experiments.

of labeled arachidonic acid and a rapid metabolism of the arachidonoyl moiety in PC, since it could be restored by a further addition of labels. The radioactivity of PE, on the other hand, was increased with incubation time, and it became higher than that of PC after 30 min incubation. The results suggest that part of the 20:4(n-6) moiety in PE may be derived from 20:4(n-6)-PC. The time-course of [1-^{14}C]20:5-(n-3) incorporation into phospholipids by PMNS from both sources resembled that of [1-^{14}C]20:4(n-6) (data not shown).

Since quantitative analyses of the diacyl-linked and ether-linked classes of PC and PE in PMNS from human blood and guinea pig peritoneal exudates have been reported (11,12), in the present study only the distribution of [1-^{14}C]20:4(n-6) and that of [1-^{14}C]20:5(n-3) among these class compositions were measured. Figure 4 shows the chromatographic resolution of alkenylacyl-, alkylacyl-, and diacyl-glyceride acetates derived from PC and PE. PC and PE from human PMNS contain more ether-linked class than diacyl-linked class, whereas the reverse is true for those from guinea pig peritoneal PMNS. It remains to prove whether the low content of ether linked PC and PE in guinea pig peritoneal PMNS is a characteristic of elicited PMNS or a reflection of the lipid pattern of guinea pig blood PMNS. It is noted that the content of alkenylacyl-PE in guinea pig peritoneal PMNS obtained in the present study is lower than that obtained by Sugiura et al. (12). Further study is required to prove whether the discrepancy is a result of the use of different time periods in collecting PMNS from guinea pig peritoneal exudates. In the present study PMNS were collected 14 hrs after intraperitoneal injection of 12% sodium caseinate solution to guinea pigs; the time period of elicitation was 2 to 4 hrs longer than that performed by Sugiura et al. (12). If ethanolamine plasmalogen is one of the endogenous sources of 20:4(n-6) in stimulated PMNS, the content of this class of PE is expected to be decreased after a longer period of elicitation.

As demonstrated in Table 1, the rate of esterification of [1-^{14}C]20:4(n-6) into diacyl-linked and ether-linked PC and PE differed. The arachidonoyl moiety in diacyl-linked PC and PE appears to be more metabolically active than the corresponding ether-linked class. It was more rapidly incorporated into the diacyl-linked PC and PE by PMNS from both sources, but it was retained to a greater extent in alkylacyl-PC and alkenylacyl-PE. After 2 hr incubation of human PMNS with [1-^{14}C]20:4-(n-6) the radioactivity in diacyl-PC and alkylacyl-PC became comparable and that in alkenyl-

TABLE 1

Distribution of [^{14}C] Arachidonic Acid in Diacyl-, Alkylacyl- and Alkenylacyl-phosphatidylcholine and -phosphatidylethanolamine*

Human PMNS

Time (Min)	Phosphatidylcholine			Phosphatidylethanolamine		
	Diacyl	Alkylacyl (%)	Alkenylacyl	Diacyl	Alkylacyl (%)	Alkenylacyl
10	83.1	16.0	0.90	62.2	12.6	25.2
20	72.5	25.3	2.21	48.6	15.4	36.0
30	67.7	29.9	2.40	38.9	14.5	46.6
60	60.3	37.1	2.60	33.3	13.3	53.4
120	54.1	43.3	2.60	26.3	13.0	60.7

Guinea Pig Peritoneal PMNS

Time (Min)	Phosphatidylcholine			Phosphatidylethanolamine		
	Diacyl	Alkylacyl (%)	Alkenylacyl	Diacyl	Alkylacyl (%)	Alkenylacyl
10	92.5	6.70	0.80	93.1	3.66	3.24
20	89.4	9.75	0.85	89.3	5.24	5.46
30	86.2	12.6	1.20	84.7	8.13	7.17
60	84.1	14.4	1.50	83.1	7.30	9.60
120	82.5	15.8	1.70	83.0	6.50	10.5

*PMNS (20 X 10^6 cells) from human blood and guinea pig peritoneal exudates were separately incubated with [1-^{14}C]20:4(n-6) (2.54 X 10^6 dpm) at 37 C at indicated time. PC and PE were purified and treated with phospholipase C as described in Materials and Methods. The amount of radioactivity in each class of the resultant diglyceride acetates is the average value from two experiments and is expressed as a percent of the total radioactivity recovered from the thin-layer plate.

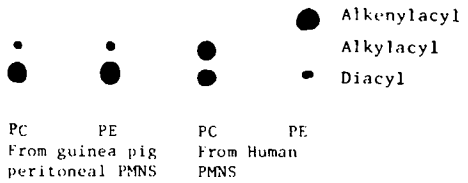

FIG. 4. TLC separation of diglyceride acetates derived from PC (1.2 μg phosphorus) and PE (1.2 μg phosphorus) isolated from human PMNS or from guinea pig peritoneal exudates. The thin-layer plate of silica gel H was first (7 cm) developed with hexane: ether (1:1, v/v), then (15 cm) with toluene (10). Spots were detected by charring the plate with 50% sulfuric acid containg 0.06% potassium dichromate.

acyl-PE comprised 61% of the total radioactivity in PE fraction. Under identical incubation conditions a small but significant amount of radioactivity was detected in alkenylacyl-PC and alkylacyl-PE. In guinea pig peritoneal PMNS, the percentage of radioactivity in alkylacyl-PC and in alkenylacyl-PE was much lower than that in human cells. The marked loss of label in PC from guinea pig peritoneal PMNS after a longer incubation period is probably accounted for by the higher contents of diacyl-linked PC in these cells than in human cells. A more rapid metabolism of the 20:4(n-6) group in the diacyl-PE than in the alkenylacyl-PE was also demonstrated in rat testis during essential fatty acid deficiency (13). Further studies are required to determine whether the transfer of arachidonoyl group from PC to PE is catalyzed by a coenzyme A dependent acyltransferase (14) or by a recently discovered transacylase in platelets (15).

The retainment of [1-^{14}C]20:4(n-6) by alkylacyl-PC and alkenylacyl-PE to a greater extent in vitro than the corresponding diacyl-linked class demonstrated in the present study is compatible with the distribution of endogenously esterified 20:4(n-6) in vivo (11,12). In human PMNS the enrichment of 20:4(n-6) in alkenylacyl-PE suggests that, in addition to PC and PI (16), ethanolamine plasmalogen may be an important endogenous source of 20:4(n-6) in stimulated cells for the synthesis of lipoxygenase products. Indeed, a loss of endogenous 20:4(n-6) from both PC and PE was identified in PMNS from human patients with chronic myelogenous leukemia after phagocytosis (17).

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REFERENCES

1. Ochi, K.; Yochimoto, T.; and Yamamoto, S. (1983) J. Biol. Chem. 258, 5754-5758.
2. Goldman, D.W.; Pickett, W.C.; and Goetzl, E.J. (1983) Biochem. Biophys. Res. Comm. 117, 282-288.
3. Takamori, K., and Yamashita, T. (1980) Inf. Imm. 29, 395-400.
4. Pember, S.O.; Barnes, K.C.; Brandt, S.J.; and Kinkade, Jr., J.M. (1983) Blood 61, 1105-1115.
5. Tou, J.-S. (1979) Biochim. Biophys. Acta 572, 307-313.
6. Bligh, E.C., and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911-917.
7. Tou, J.-S. (1978) Biochim. Biophys. Acta 531, 167-178.
8. Tou, J.-S. (1981) Biochim. Biophys. Acta 665, 491-497.
9. Waku, K.: Ito, H.; Bito, T.; and Nakazawa, Y. (1974) J. Biochem. 75, 1307-1312.
10. Rendonen, O., and Luukkonen, A. (1976) in Lipid Chromatographic Analysis, Vol. 1, 2nd Edition (Marinetti, G.V, ed.), p. 37, Marcel Dekker, New York.
11. Mueller, H.W.; O'Flaherty, J.T.; and Wykle, R.L. (1983) Fed. Proc. 42, 2230.
12. Sugiura, T.; Onuma, Y.; Sekiguchi, N.; and Waku, K. (1982) Biochim. Biophys. Acta 712, 515-522.
13. Blank, M.L.; Wykle, R.L.; and Snyder, F. (1973) Biochim. Biophys. Acta 316, 28-34.
14. Trotter, J.; Flesch, I.; Schmidt, B.; and Ferber, E. (1982) J. Biol. Chem. 257, 1816-1823.
15. Kramer, R.M., and Deykin, D. (1983) J. Biol. Chem. 258, 13806-13811.
16. Walsh, C.E.; DeChatelet, L.R.; Chilton, F.H.; Wykle, R.L.; and Waite, B.M. (1983) Biochim. Biophys. Acta 750, 32-40.
17. Smolen, J.E., and Shohet, S.B. (1974) J. Clin. Inves. 53, 726-734.

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Effects of Streptozotocin-Induced Diabetes on Microsomal Long-Chain Fatty Acyl-CoA Synthetase and Hydrolase

A.Q. DANG, F.H. FAAS* and **W.J. CARTER**, *Veterans Administration Medical Center, 300 East Roosevelt Road, Little Rock, AR 72206, and Department of Medicine, University of Arkansas for Medical Sciences, Little Rock, AR 72205*

ABSTRACT

Streptozotocin-induced diabetes significantly decreased rat liver microsomal long-chain fatty acyl-CoA (LCA-CoA) hydrolase. The decrease was observed using either palmitoyl-CoA (35 per cent, $p < 0.01$) or oleoyl-CoA (23 per cent, $p < 0.01$) as the substrate for the enzyme. Under the same conditions, diabetes did not significantly alter activity of LCA-CoA synthetase. Daily subcutaneous injections of protamine zinc insulin (10-12 units/day) into the diabetic rats returned their blood glucose to normal but only partially corrected the LCA-CoA hydrolase activity and did not effect LCA-CoA synthetase activity. The decreased LCA-CoA hydrolase and the unchanged LCA-CoA synthetase activities in the diabetic rat liver were interpreted as factors that may contribute to elevation of fatty acyl-CoA levels in the diabetic liver.
Lipids 19:578-582, 1984.

INTRODUCTION

Significant elevations of hepatic long-chain fatty acyl-CoA (LCA-CoA) levels in fasted and alloxan-induced diabetic rats were independently reported two decades ago by different laboratories (1,2). Several cellular enzymes are inhibited by the LCA-CoA thioesters such as fatty acid synthetase, acetyl-CoA carboxylase, and glucokinase (1,3). Although some of these inhibitory effects may be normal metabolic control mechanisms, increasing LCA-CoA levels in the livers of fasted or diabetic rats may be detrimental to cellular function.

LCA-CoA thioesters are synthesized by the enzyme LCA-CoA synthetase (EC 6.2.1.3) and are utilized in several ways such as acylation of glycerol 3-phosphate to form triglycerides or phosphoglycerides, acylation of cholesterol to form cholesteryl esters or β-oxidation in mitochondria to form CO_2 and H_2O. The LCA-CoA thioesters can also be hydrolyzed back to the fatty acid and CoA by the enzyme LCA-CoA hydrolase (EC 3.1.2.2). LCA-CoA hydrolase activity resides primarily in the mitochondrial and microsomal fractions of liver, with the microsomal LCA-CoA hydrolase exhibiting higher enzyme specific activity than the mitochondrial enzyme (4,5). During studies on the effects of streptozotocin-induced diabetes on acyl-CoA: glycerol 3-phosphate and acyl-CoA: lysophosphatidylcholine acyltransferases, we found that liver microsomal LCA-CoA hydrolase activity was significantly decreased in the diabetic animals, while LCA-CoA synthetase activity was not significantly changed. The results of these studies are reported in this communication.

*To whom correspondence should be addressed, at the VA address above.

MATERIALS AND METHODS

5,5'-Dithiobis-(2-nitrobenzoic acid) (DTNB), palmitoyl-CoA, oleoyl-CoA and triton WR-1339 (tyloxapol) were obtained from Sigma Chemical Company, St. Louis, Missouri. Precoated silica gel G TLC plates were purchased from Eastman Kodak Company, Rochester, New York. Streptozotocin was kindly donated by Dr. W. Dulin, Upjohn, Kalamazoo, Michigan. [1-^{14}C]Palmitic acid, [1-^{14}C]palmitoyl-CoA, Aquasol -2 and Omniflour were obtained from New England Nuclear, Boston, Massachusetts. All other chemicals and reagents were reagent grade and commercially available.

Animals and Their Treatment

Male Sprague-Dawley rats from Charles River Breeding Laboratories were maintained on a Purina Chow diet and water ad libitum. Rats were about 1½ months old and weighed about 200-220 gm (see Table 1). They were divided into three groups: Controls (17), diabetics (17), and insulin treated diabetics (9). Streptozotocin (SZ) was dissolved in cold 25 mM citrate buffer, pH 4.5, and prepared just before injection. Diabetes was induced by the IV injection of SZ into the rat's tail vein at a dose of 75 mg/kg body weight. The time between SZ injection and sacrifice was 3 weeks. In insulin treated diabetic rats, 10-12 units of protamin zinc insulin (PZI) was injected subcutaneously daily for 3 days into diabetic rats beginning 2 weeks after SZ treatment. They were sacrificed 18 hours after the last insulin

TABLE 1

Effects of SZ-Induced Diabetes on Blood Glucose and Body Weights

		Controls	Diabetes	Per cent Change	D + I
Body weights (g)	before SZ treatment	213 ± 7 (17)	202 ± 6 (17)		
	before death	308 ± 11 (17)	*230 ± 14 (17)	−25	
Blood glucose (mg/dl)	before death	120 ± 9 (17)	*447 ± 14 (17)	+273	†115 ± 21 (9)

Values are expressed as mean ± SEM.

D + I—Insulin treated diabetic rats.

*$p < 0.01$, experimental versus control; ( )—number of rats.

†Blood glucose levels before insulin treatment were 402 ± 24 mg/dl (9).

injection. Random blood glucose was estimated by using Dextrostix reagent strips read in an Ames reflectance meter. Before injecting SZ, beginning insulin treatment, and before sacrifice, blood was taken from a tail vein for glucose determination by a commercial glucose oxidase method (Sigma kit 510).

Preparation of Microsomes

Rats were killed by a blow on the head, and the entire liver was homogenized in ice-cold 0.25 M sucrose (5 ml/g wet weight liver). The homogenate was centrifuged at 1000 × g for 10 min to eliminate cell debris and nuclei. This and all subsequent steps were carried out at 4 C. The supernatant was then centrifuged at 16,000 × g for 20 minutes and the resulting supernatant centrifuged at 100,000 × g for 60 min to obtain the microsomal pellet. After the surface of the microsomal pellet was washed twice with ice-cold 0.25 M sucrose, the pellet was resuspended in 0.25 M sucrose to a protein concentration of 20 mg/ml. The microsomal suspension was divided into small portions and stored at −70 C until used. Protein was assayed by a modification of the Lowry method as described by Hartree (6).

Enzyme Assays

Fatty acyl-CoA synthetase assay: Enzyme activity was assayed as described by Marcel and Suzue (7). The reaction mixture contained 200 mM Tris-HCl, pH 7.4, 20 μg triton WR-1339, 5 mM dithiothreitol, 10 mM ATP, 4 mM $MgSO_4$, 1.25 mM CoA, 20 μM ^{14}C-palmitic acid (S.A. = 9 mCi/mmole), and 2.5 μg microsomal protein in a final volume of 0.20 ml. The reaction mixtures were incubated at 37 C for 3 min or as indicated, and the reactions stopped by adding 1 ml of 1 N H_2SO_4. Control reaction mixtures were carried out omitting CoA or by stopping the reaction at 0 min. The unreacted ^{14}C-palmitic acid was extracted with four 5 ml portions of diethylether. The aqueous phase containing the ^{14}C-palmitoyl-CoA product was transferred to counting vials. After residual ether was evaporated by a N_2 stream, 10 ml of Aquasol-2 was added to the vials and radioactivity determined.

Fatty acyl-CoA hydrolase: This enzyme was measured either by a spectrophotometric assay or radioactive tracer assay. In the spectrophotometric assay, enzyme activity was measured by following the reduction of DTNB by the thiol group of released CoA at room temperature using the Aminco DW-2 UV-Vis spectrophotometer in the split beam mode. Both reference and sample cuvettes held 1 ml of a mixture containing 100 mM Tris-HCl, pH 7.4, 0.33 mM DTNB and 25 μM palmitoyl-CoA. Reactions were started by adding 10 μl of rat liver microsomes containing 25 μg protein to the sample cuvette, unless otherwise indicated. Initial reaction rate was recorded at 412 nm for 1 to 3 minutes, and the enzyme activity was calculated based on a molar extinction coefficient of 13,600 M^{-1} cm^{-1}. Reaction rates were linear with time.

In the radioactive tracer assay, enzyme activity was assayed at 37 C. Reaction mixtures contained 100 mM Tris-HCl, pH 7.4 and 25 μM ^{14}C-palmitoyl-CoA (S.A. = 1.15 mCi/mmole) in 1 ml. Reactions were started by adding 25 μg microsomal protein and stopped 2 min later by adding 4 ml $CHCl_3$:CH_3OH (2:1, v/v) and 1 ml of 0.2 N HCl. Controls were carried out by stopping reactions at 0 min. The chloroform layer was washed twice with 4 ml of CH_3OH: 0.1 N HCl (1:1, v/v) and evaporated to dryness by a N_2 stream. The product, ^{14}C-palmitic acid, was separated from ^{14}C-palmitoyl-CoA by TLC on silica gel G plates using the solvent system: n-hexane:diethylether:acetic acid (80:20:1, v/v). After identifying the products on TLC by iodine vapor, the individual fractions were cut out, placed in scintillation vials and counted using 10 ml of omniflour cocktail.

RESULTS

Table 1 indicates that streptozotocin treatment (75 mg/kg body weight) of the rats caused an increase in blood glucose to greater than 400 mg/dl and a body weight gain that was 25 per cent less than controls. Polyuria, polyphagia and polydipsia were observed in the diabetic rats. Treatment of the diabetic rats with protamin zinc insulin (10-12 units/day) for three days corrected these symptoms and brought blood glucose levels back to normal.

Effects of Diabetes on Microsomal Long-Chain Fatty Acyl-CoA Synthetase (LCA-CoA Synthetase)

Figure 1a shows that the enzyme activity was linear with time up to 5 min of incubation. Figure 1b shows that the enzyme activity was linear with microsomal protein concentration up to 3 μg protein per reaction. For these reasons, enzyme assays were carried out using about 2.5 μg protein per assay and an incubation time of 3 min at 37 C. Similar assay conditions were used in a previous study (7). The enzyme activities in control and diabetic animals are reported in Table 2. Results of these studies indicated that the specific activity of the microsomal LCA-CoA synthetase was not significantly changed by diabetes. Insulin treatment of the diabetic rat also caused no change in enzyme activity.

Effects of Diabetes on Microsomal Long-Chain Fatty Acyl-CoA Hydrolase (LCA-CoA Hydrolase)

The specific activity of microsomal LCA-CoA hydrolase is similar to that of LCA-CoA synthetase (Table 2). Enzyme activity of the hydrolase was linear with protein concentration to 75 μg per reaction (Fig. 2). To check the specificity of the spectrophotometric assay, we also used a radioactive tracer assay which measured the conversion of [1-^{14}C]palmitoyl-CoA to [1-^{14}C]palmitic acid. As shown in Table 2, both the spectrophotometric and radioactive assays gave similar results, thus validating the spectrophotometric assay. Streptozotocin-induced diabetes caused a 25 to 37 per cent decrease in microsomal LCA-CoA hydrolase activity when palmitoyl-CoA was used as substrate, and a 23 per cent decrease when oleoyl-CoA was used as substrate. Treatment of the diabetic rats for 3 days with insulin did not correct the decreased LCA-CoA activity.

DISCUSSION

Results of this study indicate that, while

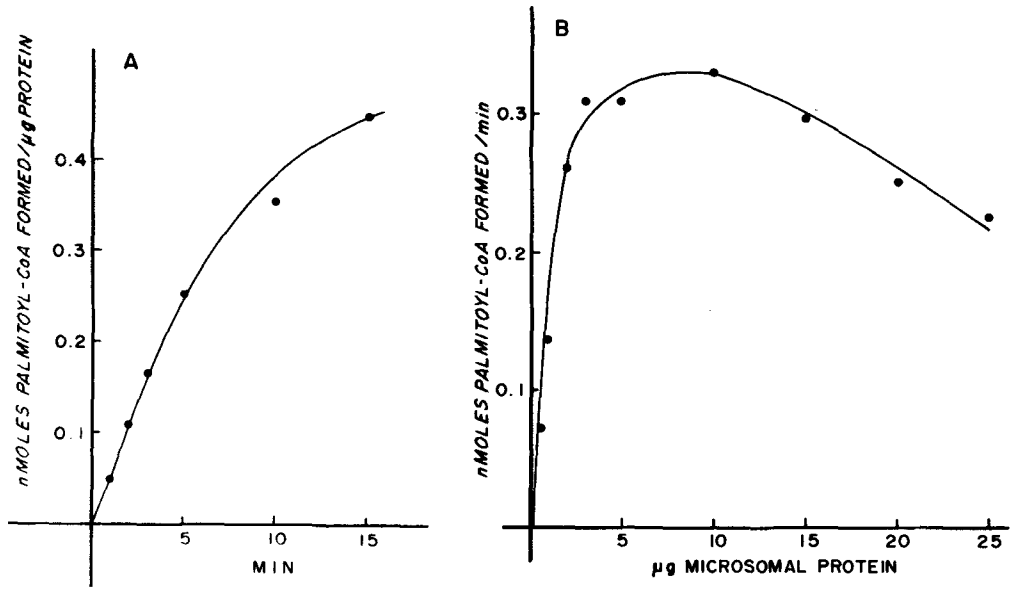

FIG. 1. Microsomal fatty acyl-CoA synthetase time and protein-dependent curves. The enzyme was assayed by following the incorporation of [1-^{14}C]palmitic acid into water soluble product. FIG. 1A. Reaction mixtures (see Materials and Methods) contained 2.5 μg microsomal protein per assay and were incubated at 37 C. At various incubation periods as indicated in the abscissa, the reaction was stopped by adding 1 ml of 1 N sulfuric acid. The unreacted substrate ([1-^{14}C]palmitic acid) was extracted with diethylether. The remaining product ([1-^{14}C]palmitoyl-CoA) in the aqueous layer was determined by a liquid scintillation spectrophotometer. FIG. 1B. Same as Fig. 1A, except that time of incubation was 3 min and various amounts of microsomal protein were present in the reaction mixtures as indicated in the abscissa.

TABLE 2

Effects of SZ-Induced Diabetes on Microsomal Fatty Acyl-CoA Synthetase and Hydrolase Activities

Enzyme	Specific Activity (nmoles/mg/min)				
	Controls	Diabetes	Per cent Change	D + I	Per cent Change
Palmitoyl-CoA synthetase	91.5 ± 3.3 (16)	82.4 ± 6.2 (15)	−10	88.7 ± 9.7 (9)	−3
Palmitoyl-CoA hydrolase					
(spectrophotometric assays)	83.5 ± 6.6 (18)	*53.0 ± 5.9 (18)	−37	62.3 ± 8.8 (9)	−25
(radioactive tracer assays)	99.6 ± 6.0 (5)	*64.5 ± 9.5 (8)	−35		
Oleoyl-CoA hydrolase	41.9 ± 1.0 (12)	*32.2 ± 1.8 (12)	−23	*30.0 ± 0.9 (5)	−25

Values are expressed as mean ± SEM.

D + I—insulin treated diabetic rats.

*$p < 0.01$, experimental versus control; ( )—number of rats.

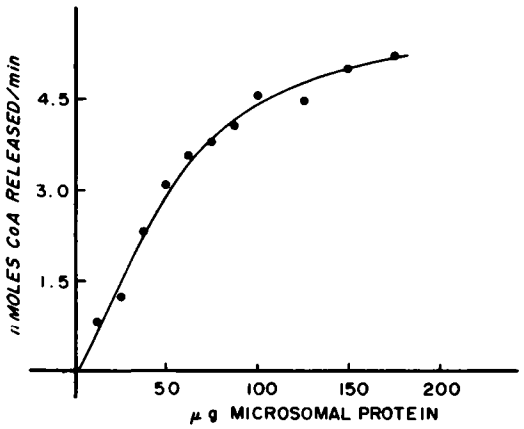

FIG. 2. Microsomal fatty acyl-CoA hydrolase. This enzyme was assayed at room temperature by using the Aminco DW-2 UV-Vis spectrophotometer, set for split beam mode. Both reference and sample cuvettes contained 1 ml of 100 mM Tris, HCl, pH 7.4, 0.33 mM 5,5'-dithiobis-(2-nitrobenzoic acid) and 25 μM palmitoyl-CoA. Reactions were started by adding 10 μl of rat liver microsomes containing various amounts of protein as indicated in the abscissa. A linear reaction rate was recorded at 412 nm, and the enzyme activity was calculated based on a molar extinction coefficient of 13,600 M^{-1} cm^{-1}.

diabetes caused very little effect on microsomal LCA-CoA synthetase activity, it consistently caused a significant decrease in microsomal LCA-CoA hydrolase activity. Although this decrease was not corrected by 3 days of insulin treatment, longer duration of treatment may be necessary for reversal of the defect.

Previous studies in fat cells have shown that LCA-CoA synthetase is regulated by insulin (8). However, studies in the perfused rat liver showed that insulin had no effect on LCA-CoA synthetase activity (9). Thus, our study demonstrating no change in liver microsomal LCA-CoA synthetase activity in the diabetic rat is consistent with this finding. Similarly, Murthy and Shipp (10) indicated that streptozotocin-induced diabetes had no effect on heart microsomal LCA-CoA synthetase activity.

In prior studies of LCA-CoA hydrolase activity, Kurooka et al. (11) showed that enzyme activity per g wet weight was significantly increased in the 16,000 g supernatant from the liver and kidney of alloxan-induced diabetic rats, provided the supernatant had been preheated to 55 C for 3 min prior to the assay. These authors found that more than 90 per cent of the increased liver LCA-CoA hydrolase activity was found in the cytoplasmic fraction. Recent studies have shown that liver LCA-CoA hydrolase activity occurs predominantly in the microsomal fraction (4,5). Thus, the difference between our finding of decreased LCA-CoA hydrolase activity in the diabetic rat and that of Kurooka et al. (11) may be attributed to differences in the cellular distribution of the enzyme studied (microsomes versus cytosol), the methods of assay (presence or absence of preheating the enzyme), the manner in which the data are expressed (enzyme activity/mg microsomal protein versus g of wet tissue) or the etiology of the diabetes (alloxan vs streptozotocin). Hashimoto and Dayton found aortic microsomal LCA-CoA hydrolase activity to be 40 per cent higher in streptozotocin-induced diabetic rats than in controls (12). Thus, microsomal LCA-CoA hydrolase activity in diabetes may also vary in different tissues.

The physiological role(s) as well as mechanism(s) for the regulation of the LCA-CoA hydrolase(s) is still unknown. It is well known that there is an increased flow of fatty acids from adipose tissue to the liver in diabetes providing increased substrate for fatty acyl-CoA formation and β-oxidation (13). Although this increased substrate flow may be the major

factor causing the increased levels of hepatic LCA-CoA thioesters in diabetes (1,2), decreased hepatic microsomal LCA-CoA hydrolase activity in diabetic animals (Table 2) also may contribute to increased LCA-CoA levels. There are undoubtedly other factors such as decreased sn-glycerol 3-phosphate concentration in the liver of starved rats (2,14) which would limit the esterification of LCA-CoA, thereby increasing LCA-CoA levels. On the other hand, increased activity of phospholipid acyl-CoA transferases in the diabetic rat (15) and increased fatty acyl-CoA β-oxidation would tend to lower LCA-CoA levels. Thus, the increased level of LCA-CoA in the liver of the diabetic rat is the result of a complicated variety of reactions which may be either stimulated or depressed by the diabetic state.

The decrease in microsomal LCA-CoA hydrolase activity in the livers of diabetic animals will influence the breakdown of added LCA-CoA used in in vitro assays of enzymes using this substrate such as acyltransferases. For this reason, care should be taken in measuring these enzymes in diabetic animals to be certain that observed effects cannot be attributed to decreased LCA-CoA hydrolase activity.

In summary, we have found decreased hepatic microsomal LCA-CoA hydrolase activity in the diabetic rat without alteration of hepatic LCA-CoA synthetase activity. This may contribute to the increased hepatic LCA-CoA levels in diabetes, and may interfere with the in vitro assay of enzymes utilizing fatty acyl-CoA substrates.

REFERENCES

1. Tubbs, P.K., and Garland, P.B. (1964) Biochem. J. 93, 550-557.
2. Bortz, W.M., and Lynen, F. (1963) Biochem. Z. 339, 77-82.
3. Tippett, P.S., and Neet, E.K. (1982) J. Biol. Chem. 257, 12846-12852.
4. Berge, R.K., and Farstad, M. (1979) Eur. J. Biochem. 95, 89-97.
5. Berge, R.K.; Vollset, S.E.; and Farstad, M. (1980) Scand. J. Clin. Lab. Invest. 40, 271-278.
6. Hartree, E.F. (1972) Anal. Biochem. 48, 422-427.
7. Marcel, L.Y., and Suzue, G. (1972) J. Biol. Chem. 247, 4433-4436.
8. Jason, J.C.; Polokoff, M.A.; and Bell, R.M. (1976) J. Biol. Chem. 251, 1488-1492.
9. Bates, E.J.; Topping, D.L.; Sooranna, S.P.; Saggerson, D.; and Mayes, P.A. (1977) FEBS Letters 84, 225-228.
10. Murthy, V.K., and Shipp, J.C. (1980) J. Mol. Cell. Cardiol. 12, 299-309.
11. Kurooka, S.; Hosoki, K.; and Yoshimura, Y. (1971) J. Biochem. 9, 247-249.
12. Hashimoto, S., and Dayton, S. (1977) Atherosclerosis 26, 289-296.
13. McGary, J.D. (1979) Diabetes 28, 517-523.
14. Declercq, P.E.; Debeer, L.J.; and Mannaerts, G.P. (1982) Biochem. J. 204, 247-256.
15. Dang, A.Q.; Faas, F.H.; and Carter, W.J. Effects of streptozotocin-induced diabetes on phosphoglyceride metabolism of the rat liver (1984) Lipids (accepted for publication).

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Fatty Liver Caused by Chronic Alcohol Ingestion is Prevented by Dietary Supplementation With Pyruvate or Glycerol

G. ANANDA RAO*, DIANA E. RILEY and EDWARD C. LARKIN, *Veterans Administration Medical Center, 150 Muir Road, Martinez, CA 94553*

ABSTRACT

Earlier studies showed that the fatty liver, caused by feeding rats the Lieber-DeCarli alcohol diet for four weeks, was prevented if the diet was supplemented with dihydroxyacetone (22 g/l), pyruvate (22 g/l) and riboflavin (2.2 g/l). In the present study, we observed that fatty liver was prevented if the alcohol diet was supplemented with glycerol and lactate (22 g/l each) and riboflavin (2.2 g/l). Hence, the prevention of alcoholic fatty liver by the dietary supplementation with dihydroxyacetone and pyruvate may not be related to their capacity to serve as hydrogen acceptors and to oxidize NADH produced during ethanol metabolism. When rats were fed the alcohol diet supplemented with either glycerol or pyruvate, the hepatic triglyceride (TG) levels were similar to those in rats pair-fed a Lieber-DeCarli control diet in which alcohol was replaced with an isocaloric amount of dextrins. Therefore, the prevention of fatty liver does not require the simultaneous presence of several supplements. Dietary dihydroxyacetone or riboflavin did not reduce alcoholic fatty liver. Supplementation of the ethanol diet with isocaloric amounts of lactate or glucose, instead of pyruvate, did not abolish the development of fatty liver but caused a marked reduction in the hepatic TG levels. Animals fed the alcohol diet consumed only small amounts of carbohydrate for long periods of time. Since the inclusion of glucose or its metabolites in the alcohol diet fed to rats caused a marked decrease in the liver TG content, it is likely that the production or prevention of fatty liver is related to carbohydrate metabolism.

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INTRODUCTION

Alcohol is the most abused drug in the United States. About 75% of all deaths caused by alcoholism are due to cirrhosis of the liver. The three common disorders produced by alcohol abuse are fatty liver, hepatitis and cirrhosis. Fatty liver is the earliest and most common response to alcohol ingestion. The degree to which it contributes to the development of more advanced stages of liver injury is unknown. If it is a requisite for the development of hepatitis and cirrhosis, then these later stages of alcoholic liver damage can be avoided by preventing fatty liver.

Previous studies have shown that the fatty liver produced in rats by feeding them a Lieber-DeCarli alcohol diet could be prevented if the diet was supplemented with a mixture of dihydroxyacetone, pyruvate and riboflavin (1,2). These compounds were considered effective since they could serve as hydrogen acceptors and oxidize the NADH which is produced during ethanol oxidation (1). In the present study, dihydroxyacetone and pyruvate were replaced by their reduced counterparts, namely glycerol and lactate, in order to determine whether the removal of NADH was directly responsible for the prevention of fatty liver.

Although Stanko et al. (1) observed that dietary supplementation with dihydroxyacetone, pyruvate and riboflavin abolished alcoholic fatty liver, it was not known whether the simultaneous presence of all three compounds was necessary. In exploratory experiments to find compounds that can prevent alcoholic fatty liver, several chemical agents were examined (1). However, they were investigated with an alcoholic dose which did not cause fatty liver. For example, rats were fed Purina Laboratory Chow and given alcohol by gastric intubation once a day for 10 days. The amount of esterified fatty acids in the livers of alcohol fed rats was 11.35 meq/100 g. This value was only slightly greater than that in controls (9.74 meq/100 g) and considerably less than that observed in rats fed either a Lieber-Decarli control (ca 15 meq/100 g) or an ethanol (ca 25 meq/100 g) diet (1). Furthermore, the differences between the hepatic lipid levels in rats fed alcohol with or without the supplements were small (for example 11.35 meq/100 g in alcohol-fed vs 11.00 meq/100 g in alcohol + pyruvate) (1). Hence, we determined the individual effect of these three substances in preventing fatty liver by giving rats a chronic dose of alcohol. These and related studies showed that fatty liver was not produced when rats were fed the Lieber-DeCarli alcohol diet supplemented with either pyruvate or glycerol. The results suggested also that the production or prevention of alcoholic

*To whom correspondence should be addressed.

fatty liver may be related to hepatic carbohydrate metabolism.

MATERIALS AND METHODS

Animals and Diets

Sprague Dawley male rats weighing ca 100 to 125 g were purchased from Bantin and Kingman Inc. (Fremont, California). They were fed a stock diet (Wayne Lab Blox; Allied Mills, Chicago, Illinois) and water ad libitum for 4 to 7 days prior to the experiment. They were housed individually in stainless steel cages having wire bottoms. Animal room was maintained at 24 C with a 40 to 50% relative humidity. It had a 12 hr dark and 12 hr light cycle. For 4 weeks, each group of 4 to 6 rats was fed one of the diets listed in Table 1. The effects of several dietary supplements in preventing alcohol-induced fatty liver were studied by four different experiments. In the first one, the effect of dihydroxyacetone, pyruvate and riboflavin was compared with that of glycerol, lactate and riboflavin. In another, the individual effect of dihydroxyacetone, pyruvate and riboflavin was examined. In the third study, inclusion of pyruvate in the diet was compared with that of glucose. In the fourth experiment, either glycerol or lactate was added to the alcohol diet and the effect on fatty liver was investigated. In order to confirm some observations, certain additives were used in more than one experiment. Rats in the alcohol group were weaned on the diet by feeding them a mixture of 1/3 alcohol and 2/3 control diets for 3 days and a mixture of 2/3 alcohol and 1/3 control diets for 4 days. From the eighth day onward, they were fed the Lieber-DeCarli ethanol diet ad libitum for four weeks. Rats in other groups were individually pair-fed to those in the alcohol group for 4 weeks.

The Lieber-DeCarli diets (control and alcohol) were purchased from Bio-Serv, Inc. (Frenchtown, New Jersey). The composition of the diets has been given in detail elsewhere (3). Dihydroxyacetone, pyruvate, glycerol and lactate were from Sigma (St. Louis, Missouri). Dextrose and riboflavin were obtained from ICN Pharmaceuticals, Inc. (Cleveland, Ohio).

Analysis of Liver TG Content

Rats were anesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/ml/300 g rat). Livers were removed, rinsed in ice-cold saline and blotted. Small portions of liver (0.3-0.5 g) were cut from the central portion of the left lobe and weighed. Liver pieces were homogenized in ice cold water (2 ml) and lipids were extracted from the homogenate by the method of Folch et al. (4). Triglycerides (TG) were separated from total lipids by thin layer chromatography, and their fatty acid methyl esters were prepared (5). Fatty acid methyl esters were analyzed by gas liquid chromatography as described earlier and quantitated by using methyl pentadecanoate as an internal standard (5).

TABLE 1

Diets Used in This Study

1. Lieber-DeCarli Control Diet
2. Lieber-DeCarli Control Diet supplemented with the following:
   a) Glycerol (22 g/l), Lactate (22 g/l) and Riboflavin (2.2 g/l)
   b) Glucose (18.6 g/l)
3. Lieber-DeCarli Alcohol Diet
4. Lieber-DeCarli Alcohol Diet Supplemented with the following:
   a) Dihydroxyacetone (22 g/l), pyruvate (22 g/l) and Riboflavin (2.2 g/l)
   b) Glycerol (22 g/l), Lactate (22 g/l) and Riboflavin (2.2 g/l)
   c) Dihydroxyacetone (22 g/l)
   d) Pyruvate (22 g/l)
   e) Pyruvate (11 g/l)
   f) Riboflavin (2.2 g/l)
   g) Glycerol (22 g/l)
   h) Lactate (22 g/l)
   i) Glucose (18.6 g/l)

RESULTS AND DISCUSSION

Animal Weight Gain and Diet Consumption

When rats were fed the Lieber-DeCarli alcohol diet containing various supplements, their weight gain and daily diet consumption were generally similar to those in the alcohol-fed controls (Tables 2-5). Although the values for these parameters differed from one experiment to another, they were comparable within each study. When the initial weights of rats were ca 100 g, they consumed ca 50 ml of liquid diet/day and gained 70-80 g in four weeks (Table 3). Similar results were obtained even when the initial weights of the animals were somewhat greater (Table 4). However, in other experiments (Tables 2 and 5), rats weighing 120-170 g consumed ca 60 ml diet/day and gained considerabley more weight (130-190 g). The reason for such differences in the weight gain between experiments is not known.

Liver TG Levels

As reported earlier (1,2), when rats were fed the alcohol diet containing dihydroxyacetone, pyruvate and riboflavin, fatty liver was prevented (Table 2). When glycerol and lactate replaced dihydroxyacetone and pyruvate res-

TABLE 2

Weight Gain, Diet Consumption and Liver Triglyceride Content of Rats Fed Ethanol Diet Supplemented with Glycerol and Lactate[a]

Diet	Initial wt g	Final wt g	Weight gain g	Diet consumption ml/day	Liver TG content mg/g
Alcohol	163.0 ± 4.7	304.3 ± 12.9	141.3 ± 13.2	61.4 ± 2.1	62.6 ± 14.1[b]
Alcohol + Dihydroxyacetone + Pyruvate + Riboflavin	163.5 ± 4.7	289.5 ± 15.1	126.0 ± 14.6	57.8 ± 1.7	18.9 ± 5.0
Alcohol + Glycerol + Lactate + Riboflavin	164.6 ± 3.5	299.0 ± 7.7	134.4 ± 7.8	56.8 ± 2.1	15.6 ± 3.7
Control	172.8 ± 2.9	330.3 ± 5.0	157.5 ± 2.3	61.5 ± 2.2	14.0 ± 3.4
Control + Glycerol + Lactate + Riboflavin	162.5 ± 4.1	320.3 ± 6.0	157.8 ± 7.9	59.9 ± 2.0	11.2 ± 1.5

[a]Values are given as mean ± SE from determinations with four rats in each diet group. In each group, rats were fed the indicated diet for 4 weeks.
[b]Triglyceride content in the livers of rats fed the alcohol diet was significantly ($p<0.01$) different from that in other groups (2-tailed t-test).

TABLE 3

Weight Gain, Diet Consumption and Liver Triglyceride Content of Rats Fed Ethanol Diet Supplemented with Dihydroxyacetone, Pyruvate or Riboflavin[a]

Diet	Initial wt g	Final wt g	Weight gain g	Diet consumption ml/day	Liver TG content mg/g
Alcohol	99.0 ± 3.4	178.4 ± 8.8	79.4 ± 7.1	47.8 ± 1.8	69.1 ± 6.7
Alcohol + Dihydroxyacetone	104.7 ± 3.3	178.7 ± 3.7	74.0 ± 5.8	48.7 ± 2.0	67.2 ± 7.2
Alcohol + Pyruvate	110.0 ± 2.9	183.0 ± 1.1	73.0 ± 2.1	47.7 ± 2.4	22.6 ± 2.4[b]
Alcohol+ Riboflavin	100.3 ± 1.9	164.3 ± 6.1	63.7 ± 8.7	48.4 ± 1.9	66.8 ± 5.6

[a]Values are given as mean ± SE from determinations with four rats in each diet group. In each group, rats were fed the indicated diet for 4 weeks.
[b]Triglyceride content in the livers of rats fed the alcohol + pyruvate diet was significantly ($p<0.001$) different from that in other groups (2-tailed t-test).

pectively, the fatty liver also was abolished (Table 2). Hence, the direct participation of dihydroxyacetone and pyruvate as hydrogen acceptors to utilize the NADH generated by alcohol metabolism may not be relevant in the prevention of alcoholic fatty liver.

Fatty liver also was abolished when rats were fed the alcohol diet containing only pyruvate as the supplement (Tables 3 and 4). Even when the pyruvate content was reduced by 50%, the amount of liver TG in alcohol-fed animals was reduced substantially (Table 4). The presence of either dihydroxyacetone or riboflavin in the diet had no effect on alcohol-induced fatty liver (Table 3). Therefore, in earlier studies (1,2), and in experiments given in Table 2, when dihydroxyacetone, pyruvate and riboflavin were used as dietary supplements to prevent alcoholic fatty liver, only pyruvate must have been active. Supplementation of the ethanol diet with glycerol alone also prevented fatty liver (Table 5). However, the inclusion of isocaloric amounts of glucose or lactate instead of glycerol did not abolish the fatty liver, although it caused a marked reduction in the liver TG content (Tables 4 and 5). These observations show that the inclusion of glucose or its metabolites in the diet causes a marked reduction in the liver TG content in rats given a chronic dose of alcohol. Such a conclusion is further supported by the results from earlier studies using the Lieber-DeCarli

TABLE 4

Weight Gain, Diet Consumption and Liver Triglyceride Content of Rats Fed Ethanol Diet Supplemented with Pyruvate or Glucose[a]

Diet	Initial wt g	Final wt g	Weight gain g	Diet consumption ml/day	Liver TG content mg/g
Alcohol	161.9 ± 0.9	229.5 ± 5.5	67.5 ± 4.7	52.2 ± 3.4	57.5 ± 5.7[b,c,d]
Alcohol + Pyruvate (22 g/l)	130.8 ± 0.7	215.8 ± 10.1	85.0 ± 5.0	47.7 ± 4.0	19.9 ± 3.2[b]
Alcohol + Pyruvate (11 g/l)	134.0 ± 0.3	206.5 ± 5.5	72.5 ± 5.4	46.3 ± 4.1	36.2 ± 5.3[c]
Alcohol + Glucose (18.6 g/l)	143.7 ± 3.4	222.3 ± 13.1	78.7 ± 10.1	48.4 ± 4.0	31.3 ± 2.9[d]

[a]Values are given as mean ± SE from determinations with five rats in each diet group. In each group, rats were fed the indicated diet for 4 weeks. Those with common superscripts are significantly different with b, $p<0.001$, and c,d, $p<0.01$ using the 2-tailed t-test.

TABLE 5

Weight Gain, Diet Consumption and Liver Triglyceride Content of Rats Fed Ethanol Diet Supplemented with Glycerol or Lactate[a]

Diet	Initial wt g	Final wt g	Weight gain g	Diet consumption ml/day	Liver TG content mg/g
Alcohol	152.2 ± 1.4	284.0 ± 7.1	131.8 ± 5.8	61.4 ± 1.8	78.1 ± 7.2[b]
Alcohol + Glycerol	126.2 ± 0.7	269.1 ± 8.7	142.9 ± 8.7	54.5 ± 1.9	18.5 ± 2.8
Alcohol + Lactate	131.4 ± 0.5	287.2 ± 14.8	155.6 ± 13.1	58.7 ± 1.7	37.1 ± 4.6
Alcohol + Glucose	140.5 ± 1.3	278.0 ± 15.6	137.7 ± 14.9	60.2 ± 0.6	39.9 ± 2.9
Control + Glucose	121.4 ± 0.8	311.1 ± 13.2	185.7 ± 10.3	63.4 ± 1.9	19.1 ± 2.4

[a]Values are given as mean ± SE from determinations with six rats in the alcohol and alcohol + glucose diet groups and five rats in the remaining groups. In each group, rats were fed the indicated diet for 4 weeks.
[b]Triglyceride content in the livers of rats fed the alcohol diet was significantly ($p<0.001$) different from that in other diet groups.

alcohol diet containing various levels of fat (6). Fatty livers were observed only when the level of fat in the diet was 35% of the total calories or higher. If the fat content of diet was 25% of calories or lower, the hepatic TG contents in alcohol-fed animals were reduced to levels (18-23 mg/g) observed in rats fed a control diet devoid of alcohol (6). In these instances, when the level of fat in the diet was reduced, the carbohydrate content was increased correspondingly and fatty liver was not produced. However, the effect of increased carbohydrate content of the alcohol diet in reducing or preventing fatty liver appears to be related to the level of protein in the diet. The protein content of the Lieber-DeCarli alcohol diet is 18% of total calories (3). In studies in which the protein content was reduced to 12.5% of total calories and the carbohydrate level was increased correspondingly, the extent of fatty liver was not affected (6).

Carbohydrate Deprivation in Rats Given a Chronic Dose of Alcohol

In many investigations of alcohol-induced fatty liver, animals have been fed the Lieber-DeCarli liquid diets (1-3, 5-7). It has generally been considered that fatty liver was produced by the chronic ingestion of alcohol in spite of feeding a nutritionally adequate diet (7,8). The Lieber-DeCarli control diet was suggested to be nutritionally adequate since when fed ad libitum, it promoted growth of rats in a way comparable to that by other commercial diets

(3). However, unlike the control diet, it is doubtful whether the alcohol diet provides a nutritionally adequate dietary regimen. The consumption of the alcohol diet by rats is reduced by ca 50% when compared to that of the control diet when fed ad libitum (5). Therefore, not only the daily caloric intake but also the consumption of essential dietary ingredients is reduced markedly when alcohol is included in the diet. Such a restriction in the diet affects the rate of growth of animals fed the alcohol and control liquid diets. When rats are fed the ethanol diet ad libitum, their growth rates are comparable to those of pair-fed controls (3). However, when compared to rats fed the control diet ad libitum, the weight gain of alcohol-fed rats is small (5).

The caloric contribution of carbohydrate in many normal diets is high (62-75%) (3). It is somewhat low (47%) in the Lieber-DeCarli control diet (3). It is reduced further to only 3% or 11% in alcohol diets depending on the level of fat. Hence, animals given the ethanol diet for long periods are deprived of adequate amounts of carbohydrates. As a consequence of such a dietary regimen, the glycogen content of livers is reduced (9,10). Even after feeding rats the alcohol diet for only 3 days, the liver glycogen levels are reduced by 50% (9). The liver glycogen content has also been found to be markedly reduced as compared to the controls when rats were fed an alcohol diet having a composition different from that of the Lieber-DeCarli diet (11). The glycogen content of alcoholic fatty livers is low (56 ± 20 μmole as glucose/g compared to 318 ± 48 μmole as glucose/g in normals) (10). Such reduced levels have been reported in alloxan-diabetes (12). Hence, rats fed the Lieber-DeCarli alcohol diet have low levels of glycogen in the liver for prolonged periods during the development of fatty liver. Whether such reduced levels of glycogen have a role in promoting hepatic TG accumulation is not known. The various compounds (glucose, pyruvate, lactate, dihydroxyacetone and glycerol) which were used as dietary additives can be converted to glycogen in the liver. Whether the synthesis of glycogen from these precursors occurs in alcohol-fed animals remains to be examined. Due to the generation of NADH during ethanol oxidation, pyruvate may most likely be converted to lactate rather than glycogen. If the supplements are metabolized by the liver, a sparing effect on the glycogen level is likely in the livers of alcohol-fed rats.

Of the many substances studied as supplements, only pyruvate and glycerol were capable of preventing alcoholic fatty liver (Tables 3-5). Addition of glucose or lactate to the alcohol diet reduced the liver TG levels by ca 50% (Tables 4 and 5). Unlike glycerol, dihydroxyacetone was not effective in abolishing alcoholic fatty liver (Tables 3 and 5). Prior to their metabolism, both these compounds need to be converted to their phosphorylated derivatives. Glycerol kinase, which catalyses the production of glycerol 3-phosphate from glycerol and ATP, also acts on dihydroxyacetone to yield dihydroxyacetone phosphate. Moreover, with the kinase isolated from rat livers, dihydroxyacetone was found to be phosphorylated at a rate about twice that of glycerol (13). Hence, if glycerol was active due to its metabolism in the liver, it is surprising that dihydroxyacetone was not effective in preventing fatty liver. Whether the difference in their efficiency is related to their absorption by the intestinal mucosa remains obscure. It is also surprising that an isocaloric amount of glucose was not as effective as its metabolite, pyruvate. Whether this phenomenon is due to the mobilization of some fraction of glucose from liver to extra-hepatic tissues is unknown.

Inclusion of pyruvate or glycerol (22 g/l) in the alcohol diet provided only 74 additional cals/l diet. Their presence caused a small increase (6%) in the carbohydrate calories and prevented hepatic accumulation of TG. These additives also caused a decrease in the caloric contribution of ethanol from 35.5% to 33%. Such a small decrease in the proportion of alcohol calories may not be related to the abolition of fatty liver. This is evident from the results of earlier studies in which rats were fed diets containing different alcohol calories (36% (7), 34% (2) or 33% (1)). The extent of fatty liver as indicated by the TG contents was similar in these instances. In spite of this small difference in the percentage of alcohol calories, the amount of alcohol consumed per day is the same whether rats were fed ethanol diets with or without the supplements. However, when alcohol ingestion caused fatty liver, it was avoided by the presence of small amounts of metabolites. The mechanism by which glucose or its derivatives affect the hepatic TG levels in rats given a chronic dose of alcohol is not known. It is likely that the normal metabolism of carbohydrates is intimately associated with the production of lipoproteins which are necessary for the mobilization of fat. The role of carbohydrates in the production and prevention of alcohol-induced fatty liver requires further investigation.

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REFERENCES

1. Stanko, R.T.; Mendelow, H.; Shinozuka, H.; and Adibi, S.A. (1978) J. Lab. Clin. Med. 92, 228-235.
2. Goheen, S.C.; Pearson, E.E.; Larkin, E.C.; and Rao, G.A. (1981) Lipids 16, 43-51.
3. Lieber, C.S., and DeCarli, L.M. (1982) Alcoholism: Clin. Exp. Res. 6, 523-531.
4. Folch, J.; Lees, M.; and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497-509.
5. Goheen, S.C.; Larkin, E.C.; Manix, M.; and Rao, G.A. (1980) Lipids 15, 328-336.
6. Lieber, C.S., and DeCarli, L.M. (1970) Am. J. Clin. Nutr. 23, 474-478.
7. Lieber, C.S.; Jones, D.P., and DeCarli, L.M. (1965) J. Clin. Invest. 44, 1009-1021.
8. DeCarli, L.M., and Lieber, C.S. (1967) J. Nutr. 91, 331-336.
9. Lefevre, A.; Adler, H., and Lieber, C.S. (1970) J. Clin. Invest. 49, 1775-1782.
10. Rao, G.A.; Riley, D.E.; Jarratt, B.A.; and Larkin; E.C. (1984) IRCS Med. Sci. 12, 248-249.
11. Winston, G.W., and Reitz, R.C. (1980) Life Sci. 26, 201-209.
12. Kalkhoff, R.K.; Hornbrook, K.R.; Burch, H.B.; and Kipnis, D.M. (1966) Diabetes, 15, 451-456.
13. Bublitz, C., and Kennedy, E.P. (1954) J. Biol. Chem. 211, 951-961.

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Changes in the Structure of Soybean Triacylglycerols Due to Heat

HIROMI YOSHIDA and **J.C. ALEXANDER***, *Department of Nutrition, College of Biological Science, University of Guelph, Guelph, Ontario, Canada N1G 2W1*

ABSTRACT

Commercially refined soybean oil was heated at 180 C for 50, 70 or 100 hr with aeration. The triacylglycerol fractions separated from the oils by column chromatography on silica gel were fractionated further by silicic acid thin layer chromatography (TLC), and species compositions were determined by argentation TLC and lipase hydrolysis. There was a decrease in the absolute amounts of the triacylglycerols with longer heating periods, such as 21% for 50 hr and 42% for 100 hr. Relatively large changes occurred in the proportion of the molecular species of the triacylglycerols during heating; there was an increase in the more saturated species, 1-4 double bonds, and a decrease in species containing 5-7 double bonds. Although these changes occurred in the percentage of each triacylglycerol species, the positional distribution of the fatty acids in the 2-position remained virtually unchanged throughout heating. Oleic and linoleic acids were commonly found in the 2-position of the acylglycerol moiety, whereas most of the palmitic and stearic acids favored esterification in the 1- and 3-positions. The results indicate that unsaturated fatty acids located in the 2-position are protected significantly from thermal oxidative decomposition.

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INTRODUCTION

Vegetable oils are a complex mixture of molecular species of triacylglycerols (1-5), containing from 70-95% of unsaturated fatty acids (6,7). These acids, especially linoleic and linolenic, are much more prone to degradation during heating than the saturated fatty acids. Chemical reactions such as thermal and oxidative decompositions produce both volatile and nonvolatile products, which can have adverse effects on experimental animals (8-11). Many decomposition products, both volatile (12,13) and nonvolatile (13-17), have been identified during the last 20 years. However, no studies had been reported on the effects of heating with aeration on the composition of triacylglycerol species.

Fats are susceptible to thermal oxidation even when subjected to the conditions used for food processing and deep frying (18,19). Substantial decreases in the amount of triacylglycerol have been observed in oils heated in the laboratory at 180 C with aeration (20), a temperature comparable to restaurant deep-frying. In this study we investigated the structures of molecular species of triacylglycerols in fats before and after heating to assess their stability to thermal oxidation when subjected to frying temperatures.

MATERIALS AND METHODS

Preparation of Heated Soybean Oils

Commercially available refined soybean oil was thermally oxidized at 180 C for 50, 70 or 100 hr in the presence of air (0.11-0.16 ml/min/g), using the apparatus described in a previous report (20).

Determination of Triacylglycerols

Unheated and thermally oxidized oils were separated by one dimensional TLC on Silica Gel G plates activated at 120 C for 3 hr immediately before use. Solvent systems used were petroleum ether/diethyl ether (70:30, v/v) and n-hexane/benzene (40:60, v/v). The plates were exposed to iodine vapor to visualize the triacylglycerol band. The appropriate band was scraped from the plate, and the triacylglycerols were recovered from the adsorbent by extraction with 10% methanol in diethyl ether. The content of triacylglycerol was estimated by the ester bond determination (21), and methyl palmitate was used for the calibration curve.

Isolation of Triacylglycerols

The thermally oxidized oils were separated into two fractions by column chromatography on silica gel by the method of Billek et al. (22), with minor modifications (20). Elution with 150 ml of 20% isopropyl ether in n-hexane obtained the triacylglycerols (fraction I) which were relatively nonpolar. With 150 ml of 60% isopropyl ether in n-hexane, and finally 200 ml of diethyl ether, the polar components (fraction II) were collected. The proper separation and triacylglycerol contents were verified by TLC. Along with the triacylglycerols, fraction I contained part of the unsaponifiable matter which was removed by TLC as described

*To whom correspondence should be addressed.

earlier. The unheated oil was exposed to the same fractionation procedures as above, to isolate the triacylglycerols.

Triacylglycerol Species Analysis

Silver nitrate-silica gel TLC was used to fractionate the total triacylglycerols into their various species according to the degree of unsaturation. The TLC plates were prepared by the method of Roche et al. (23). Each sample was separated with solvent systems consisting of 0.8% to 5% methanol in chloroform, depending on the polarity of the triacylglycerols (24). Individual bands were visualized under ultraviolet light after being sprayed with 2', 7'-dichlorofluorescein (0.1% in ethanol) and recovered from the plate by extraction with 10% methanol in diethyl ether, followed by diethyl ether. Quantitative determination of the amount, as well as composition of each band, was carried out by the addition of a known weight (10 to 100 g) of methyl heptadecanoate as an internal standard. The triacylglycerols were converted into fatty acid methyl esters by heating them with boron trifluoride/methanol (25), and analyzed by GLC. A Hewlett-Packard Model 5840A gas chromatograph equipped with a flame ionization detector and an electronic integrator for quantitation of peak areas was used. The other GLC conditions were as described previously (20).

Lipase Hydrolysis

The hydrolysis technique was similar to the general procedure described by Mattson and Volpenhein (26) scaled down to a semi-micro method. At least 2 mg of triacylglycerol (from more than one TLC plate if necessary) was suspended in 1 ml of Tris buffer (pH 7.6) containing 0.25 M $CaCl_2$, and 8 mg of lipase were added. The reaction was carried out in a water bath at 37 C with vigorous stirring for 13 min. Then the mixture was poured into diethyl ether over anhydrous sodium sulfate and filtered. The ether solution was concentrated to a small volume under a stream of nitrogen. Residual 2-monoacylglycerols were separated by TLC on Silica Gel G with n-hexane/diethyl ether/acetic acid (50:50:1, v/v/v). The appropriate band was scraped directly into an ampule and esterified by heating it with boron trifluoride/methanol (25). Fatty acid composition of the methyl esters was determined by GLC.

RESULTS

The relative contents of the residual triacylglycerols in the heated oils as isolated by preparative TLC were determined by the ester bond method, and the value for the fresh unheated oil was normalized to 100. With the progress of heating, the amounts of triacylglycerols gradually decreased by 21.5% after 50 hr, 29.2% after 70 hr and 42.4% after 100 hr.

The extent of the variations of molecular species in triacylglycerols was determined by quantitation with argentation TLC. The composition of bands was calculated from GLC data (24,27) on the basis of their fatty acid components (Table 1). Saturated fatty acids are denoted as S and include palmitic (16:0) and stearic (18:0) acids. Unsaturated fatty acids, oleic (18:1), linoleic (18:2) and linolenic (18:3), are designated as monoene (M), diene (D) and triene (T), respectively. No species containing two ro more linolenic acid units (T_2 or T_3) were detected in the unheated oil. The species containing three saturated fatty acids (S_3) was only a trace component (<0.09%) after 70 hr of heating. In all, fifteen different molecular species were detected by TLC, but a few containing linolenic acid (SDT, MDT and D_2T) became very small components with 100 hr of heating (Table 1). Conversely, most species containing saturated acids (S_2M, SM_2, S_2D, SMD, SD_2 and SMT) gradually increased by a range from 1.4% for SD_2 to 5.1% for SMT. Two species containing monoene and no saturate, (M_3 and M_2D) also increased by 4.9% and 5.1%, whereas two others (MD_2 and M_2T) decreased by 2.5% and 3.7% respectively. D_3 showed the greatest change with a loss of 9.6%.

Figure 1 shows selected data representing 1 to 7 double bonds in the triacylglycerol species. In general, as the heating period was extended there was an increase in the percentage of all species containing saturated fatty acids (maximum of 3 double bonds). The species representing 4 double bonds (M_2D) increased with heating in spite of an erratic response indicating reversal after 70 hr. In contrast, the species containing 5, 6 and 7 double bonds all showed substantial declines.

In order to obtain information on isomer composition, the distributions of fatty acids in the 2-position were determined by lipase hydrolysis, and the remainder of each fatty acid was esterified in the 1 and 3-positions. The results on total triacylglycerol fractions are summarized in Table 2. Very little of the saturated fatty acids were in the 2-position in the soybean oil, but increases were apparent after 100 hr of heating. More than a third of 18:1 and 18:2 remained in the 2-position after heating with no appreciable change. For 18:3 heating caused an increase in the amount in the 2-position above the original 22%.

TABLE 1

Concentrations of Triacylglycerol Species from Thermally Oxidized Soybean Oils (wt %)[a]

Triacylglycerol species[b]	No. of double bonds	Heating period (hr) 0	50	70	100	Δ[d]
S_2M	1	2.3 ± 0.2	3.1 ± 0.3	3.5 ± 0.3	6.7 ± 0.4	4.4
SM_2	2	0.7 ± 0.1	1.2 ± 0.2	1.9 ± 0.2	3.1 ± 0.3	2.4
S_2D	2	5.8 ± 0.5	6.7 ± 0.4	7.5 ± 0.5	9.2 + 0.5	3.4
M_3	3	4.1 ± 0.2	7.0 ± 0.3	7.4 ± 0.4	9.0 ± 0.4	4.9
SMD	3	4.3 ± 0.2	5.9 ± 0.3	6.7 ± 0.3	7.4 ± 0.4	3.1
M_2D	4	11.2 ± 0.9	14.3 ± 1.3	17.2 ± 1.2	16.3 ± 1.3	5.1
SD_2	4	11.9 ± 1.1	12.3 ± 1.0	13.6 ± 1.2	13.3 ± 1.2	1.4
SMT	4	1.3 ± 0.1	2.0 ± 0.1	3.4 ± 0.2	6.4 ± 0.3	5.1
MD_2	5	12.3 ± 1.1	10.8 ± 1.0	9.6 ± 1.0	9.8 ± 1.0	-2.5
M_2T	5	14.0 ± 0.9	13.1 ± 1.1	11.4 ± 0.9	10.3 ± 0.9	-3.7
D_3	6	14.5 ± 0.8	12.2 ± 0.7	10.3 ± 0.7	4.9 ± 0.2	-9.6
SDT	5	4.2 ± 0.3	3.7 ± 0.2	2.8 ± 0.2	1.7 ± 0.2	-2.5
MDT	6	4.7 ± 0.3	3.1 ± 0.3	2.0 ± 0.1	0.8 ± 0.1	-3.9
D_2T	7	8.7 ± 0.4	4.6 ± 0.3	2.7 ± 0.2	1.1 ± 0.1	-7.6
Categorical Summary[c]	A	41.6 ± 1.2	52.5 ± 1.4	61.2 ± 1.5	71.4 ± 1.7	
	B	58.4 ± 1.6	47.5 ± 1.5	38.8 ± 1.3	28.6 ± 1.0	

[a]Each value represents the mean of triplicate determinations ± SEM.

[b]Abbreviations: S, saturated; M, monoenoic; D, dienoic; T, trienoic acid moieties of triacylglycerols, e.g., SMD, a triacylglycerol containing one saturated, one monoenoic and one dienoic fatty acid; D_3, trilinolein.

[c]Total percentages of triacylglycerol species: A = the species containing 1-4 double bonds, B = the species containing 5-7 double bonds.

[d]Gain or loss for 100 hr heating relative to unheated oil.

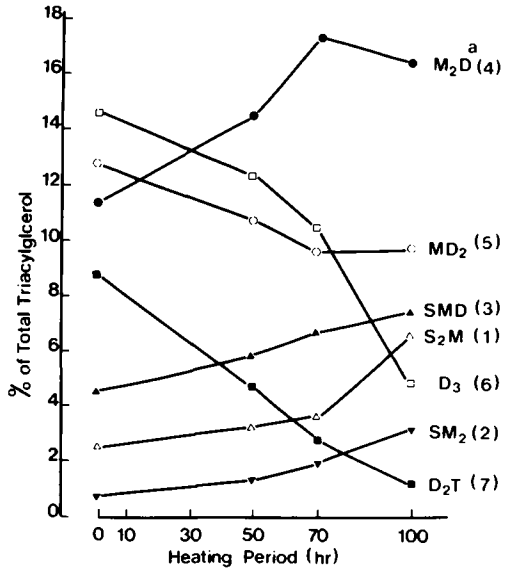

FIG. 1. Changes in the amount of selected molecular species of the triacylglycerols in the thermally oxidized soybean oils (representing 1-7 double bonds). [a]Abbreviations of triacylglycerol species: see Table 1.

For comparison, selected data for individual triacylglycerol species isolated from unheated oil and oil heated 70 hr are shown in Table 3. This detailed information allows us to see that the specific distribution patterns for the individual fatty acids changed little due to heating. In the absence of higher unsaturation, oleic acid was highly concentrated in the 2-position (S_2M and SM_2). However, when diene was present it dominated the 2-position at the expense of monoene or triene. The saturates were consistently low in this position.

DISCUSSION

Fatty acid contents of soybean oil triacylglycerols at 0, 50, 70 and 100 hr of heating have been reported (20). Distribution of fatty acids in triacylglycerols can have an influence on their rate of autoxidation (28-30), but the effect of acylglycerol structure on the rate of thermal oxidation had not been investigated. It is indefinite whether hydroperoxides are formed at frying temperatures of 180 C and above, since hydroperoxides of lipids are known to be heat-labile, and peroxides produced early during heating are unstable and break down to form thermal decomposition products (31,32). However, there is no information about their presence, or the kinetics that control their decomposition (33).

Extensive thermal oxidation of triacylglycerols occurs in soybean oil during heating (19,20,34), and the amount of triacylglycerols

TABLE 2

Lipase Hydrolysis of Total Triacylglycerol Isolated from Thermally Oxidized Soybean Oils, Showing Weight Per Cent Esterified in 2-Position[a,b]

Fatty acid	Heating period (hr)			
	0	50	70	100
16:0	1.9 ± 0.3	2.1 ± 0.3	2.0 ± 0.3	5.1 ± 0.4
18:0	2.8 ± 0.7	2.6 ± 0.6	2.0 ± 0.7	8.3 ± 0.6
18:1	35.8 ± 0.5	37.8 ± 0.5	37.7 ± 0.4	39.7 ± 0.5
18:2	42.5 ± 0.6	42.8 ± 0.5	43.8 ± 0.6	43.2 ± 0.6
18:3	21.8 ± 0.4	27.0 ± 0.3	28.6 ± 0.6	26.3 ± 0.4

[a]Per cent esterified in 2-position $= \frac{\%\text{ in 2-monoacylglycerol}}{3 \times \%\text{ in triacylglycerol}} \times 100.$

[b]Each value represents the mean of triplicate determinations ± SEM. Minor components have been omitted (<0.09).

TABLE 3

Lipase Hydrolysis of Individual Triacylglycerol Species Isolated from Thermally Oxidized Soybean Oils, Showing Weight Per Cent of Fatty Acids Esterified in 2-Position[a]

Heating Period (hr)	Triacylglycerol species[b]	No. of double bonds	Fatty acid				
			16:0	18:0	18:1	18:2	18:3
0	S_2M	1	10.3 ± 1.0	4.2 ± 0.2	85.5 ± 2.0		
	SM_2	2	3.7 ± 0.4	—	96.3 ± 2.1		
	S_2D	2	8.2 ± 0.6	9.8 ± 0.7		82.0 ± 2.0	
	SMD	3	2.5 ± 0.1	—	29.1 ± 1.2	68.4 ± 1.8	
	MD_2	5			12.6 ± 1.0	87.4 ± 2.3	
	D_2T	7				83.6 ± 2.1	16.4 ± 1.2
70	S_2M	1	11.2 ± 1.3	4.1 ± 0.3	84.7 ± 2.3		
	SM_2	2	3.6 ± 0.6	—	96.4 ± 2.5		
	S_2D	2	8.4 ± 0.7	10.2 ± 1.3		81.4 ± 2.3	
	SMD	3	2.7 ± 0.3	—	29.6 ± 1.6	67.7 ± 2.0	
	MD_2	5			13.5 ± 1.5	86.5 ± 2.5	
	D_2T	7				84.2 ± 2.3	15.8 ± 1.3

[a]Each value represents the mean of triplicate determinations ± SEM. Minor components have been omitted (<0.09).

[b]Abbreviations: See Table 1.

decreases substantially. However, residual triacylglycerols were still major components of the heated oils. The decrease in triacylglycerols was accompanied by extensive changes in molecular species concentrations (Fig. 1), which were related to the fatty acid composition of the triacylglycerols. The patterns depended on the degree of unsaturation, reflected in the total number of double bonds in the moieties (Table 1). For the unheated oil, all triacylglycerol species containing from one to four double bonds (S_2M-SMT) contributed approximately 42%, whereas those containing more than four double bonds (MD_2-D_2T) were approximately 58%. With the progress of heating, the percentage of the former increased substantially, such as 53% at 50 hr and 71% at 100 hr, whereas that of the latter dramatically decreased to about 47% at 50 hr down to 29% at 100 hr of heating. Species with more than one D in the structure would have in addition to the high level (more than 80%) in the 2-position, extra D in the unprotected 1 and 3-positions resulting in a higher rate of degradation. This drop in the number of double bonds is confirmed by lower iodine values for thermally oxidized fats (10).

When the percentages of the fatty acids remaining in the 2-position of the residual triacylglycerols (Table 2) were calculated for the dominant types, there were no major changes in the proportion of esters as a result of heating and aerating the oil. Rates of autoxidation increase directly with the number of double bonds in fatty acids (30,35). However, Raghuveer and Hammond (28) suggested that

acylglycerol structure might affect the relative rates of oxidation of acyl groups in a triacylglycerol. They indicated that acyl groups in the sn-1 and 3 positions should oxidize faster than those in sn-2. The data presented here show that the distribution of the fatty acids in the 2-position of several acylglycerol species remained nearly constant during large changes in the proportion of these species. This suggests that fatty acids in the 1- and 3-positions were more susceptible to oxidation, and that the polyunsaturated fatty acids can be conserved significantly from thermal oxidative decomposition if they are located in the 2-position. This observation agrees with the recent conclusions of Wada and Koizumi (36).

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REFERENCES

1. Brockerhoff, H., and Yurkowski, M. (1966) J. Lipid Res. 7, 62-64.
2. Lindgvist, B.; Sjogren, I.; and Nordin, R. (1974) J. Lipid Res. 15, 67-73.
3. Wada, S.; Koizumi, C.; and Nonaka, J. (1977) Yukagaku 26, 95-99.
4. El-Hamdy, A.H., and Perkins, E.G. (1981) J. Am. Oil Chem. Soc. 58, 867-872.
5. Petersson, B.; Podlaha, O.; and Toregard, B. (1981) J. Am. Oil Chem. Soc. 58, 1005-1009.
6. Langstraat, A. (1976) J. Am. Oil Chem. Soc. 53, 241-247.
7. Industry News, (1980) J. Am. Oil Chem. Soc. 57, 264A-268A.
8. Akiya, T., and Shimizu, T. (1965) Yukagaku 14, 520-522.
9. Poling, C.E.; Warner, W.D.; Mone, P.E.; and Rice, E.E. (1962) J. Am. Oil Chem. Soc. 39, 315-320.
10. Alexander, J.C. (1978) J. Am. Oil Chem. Soc. 55, 711-717.
11. Alexander, J.C. (1981) J. Toxicol. Environ, Health 7, 125-138.
12. Kawada, K.; Krishnamurthy, R.G.; Mookherjee, B.D.; and Chang, S.S. (1967) J. Am. Oil Chem. Soc. 44, 131-135.
13. Chang, S.S.; Peterson, R.T.; and Ho, C.-T. (1978) J. Am. Oil Chem. Soc. 55, 718-727.
14. Artman, N.R. (1969) in Advances in Lipid Research (Paoletti, R. and Kritchevsky, D., eds) Vol. 7, pp. 245-330, Academic Press Inc., New York, NY.
15. Perkins, E.G., and Wantland, L.R. (1973) J. Am. Oil Chem. Soc. 50, 459-461.
16. Waltking, A.E.; Serry, W.E.; and Bleffert, G.W. (1975) J. Am. Oil Chem. Soc. 52, 96-100.
17. Come, N.; Constantin, M.I.; and Emtressangles, B. (1981) Lipids 16, 8-16.
18. Paulose, M.M., and Chang, S.S. (1973) J. Am. Oil Chem. Soc. 50, 147-154.
19. Meltzer, J.B.; Frankel, E.N.; Bessler, T.R.; and Perkins, E.G. (1981) J. Am. Oil Chem. Soc. 58, 779-784.
20. Yoshida, H., and Alexander, J.C. (1982) Nutr. Rep. Int. 26, 655-665.
21. Renkonen, O. (1961) Biochem. Biophys. Acta 54, 361-362.
22. Billek, G.; Guhr, G.; and Waibel, J. (1978) J. Am. Oil Chem. Soc. 55, 728-733.
23. de la Roche, I.A.; Weber, E.J.; and Alexander, D.E. (1971) Lipids 6, 537-540.
24. Blank, M.L.; Verdino, B.; and Privett, O.S. (1965) J. Am. Oil Chem. Soc. 42, 87-90.
25. Morrison, W.R., and Smith, L.M. (1964) J. Lipid Res. 5, 600-608.
26. Mattson, F.H., and Volpenhein, R.A. (1961) J. Lipid Res. 2, 58-62.
27. Privett, O.S., and Nutter, L.J. (1967) Lipids 2, 149-154.
28. Raghuveer, K.G., and Hammond, E.G. (1967) J. Am. Oil Chem. Soc. 44, 239-243.
29. Catalano, M.; de Felice, M.; and Sciancalepore, V. (1975) Ind. Aliment. (Pinerolo, Italy) 14, 89-92.
30. Fatemi, S.H., and Hammond, E.G. (1980) Lipids 15, 379-385.
31. Frankel, E.N.; Evans, C.D.; and Cowan, J.C. (1960) J. Am. Oil Chem. Soc. 37, 418-424.
32. Perkins, E.G. (1967) Food Technol. 21, 611-616.
33. Selke, E.; Frankel, E.N.; and Neff, W.E. (1978) Lipids 13, 511-513.
34. Waltking, A.E., and Zmachinski, H. (1970) J. Am. Oil Chem. Soc. 47, 530-534.
35. Holman, R.T., and Elmer, O.C. (1947) J. Am. Oil Chem. Soc. 24, 127-129.
36. Wada, S., and Koizumi, C. (1983) J. Am. Oil Chem. Soc. 60, 1105-1109.

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The Effects of Streptozotocin Diabetes on Tissue Specific Lipase Activities in the Rat

TAKAHIDE NOMURA[a,*], YASUMICHI HAGINO[a], MINEHIRO GOTOH[b], AKIHISA IGUCHI[b] and NOBUO SAKAMOTO[b], [a]*Department of Pharmacology, and* [b]*The Third Department of Internal Medicine, Nagoya University School of Medicine, Nagoya 466, Japan*

ABSTRACT

Fasting in normal rats produced a fall in hepatic triglyceride lipase (H-TGL) activity as well as lipoprotein lipase (LPL) activities of adipose tissue and psoas minor muscle. On the other hand, LPL activities of heart and diaphragm were not decreased by fasting; the former, in fact, was increased significantly. Changes in tissue specific lipase activity caused by withdrawal of insulin from insulin-treated diabetic animals paralleled in direction the changes induced by starvation of normal rats. Furthermore, it was shown in the present paper that the tissue specific lipase activity of diabetic rats became stuck in the starve phase of the starve-feed cycle regardless of dietary intake. The changes of the tissue specific lipase activities, especially of liver, adipose tissue and heart, appeared to coincide with those of plasma insulin levels. These results strongly suggest that the tissue specific lipase system is under hormonal regulation by insulin. Streptozotocin diabetes produced hypertriglyceridemia. The possible mechanism of the hypertriglyceridemia in diabetic animals was discussed in connection with the role of the tissue specific lipase system in the serum triglyceride metabolism.
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INTRODUCTION

Hypertriglyceridemia, a risk factor for premature atherosclerosis (1), may result from decreased use of plasma triglyceride by tissues. The removal of triglyceride is thought to be mediated by triglyceride lipase (TGL) (2). It is known that heparin releases two kinds of lipolytic activities from tissues: hepatic triglyceride lipase (H-TGL) and extrahepatic lipoprotein lipase (LPL) (3,4). Further investigations on the origin of these lipases have revealed that both LPL and H-TGL components are heterogeneous; the former resides chiefly in adipose tissue, skeletal muscle, heart and mammary tissue (5,6), and the latter in the liver, ovary and adrenal gland in the rat (7). Therefore, it has been suspected that the lipase activity of each tissue changes differently as a result of alteration in the nutritional and hormonal environment (6). In other words, to understand the physiological role of the triglyceride lipase in serum triglyceride metabolism, it is necessary to study the specific tissue lipase. Nevertheless, so far, the significance of H-TGL and LPL activities in post heparin plasma has been discussed for the elucidation of the mechanism of hypertriglyceridemia in various conditions (8-10).

The aim of the present study was to investigate the effects of fasting and streptozotocin (STZ) diabetes on TGL activities of different tissues (liver, adipose tissue, psoas minor muscle, heart and diaphragm) and clarify the significance of the lipase system in the metabolism of serum triglyceride.

*To whom correspondence should be addressed. Present address: Dr. Takahide Nomura, Department of Pharmacology, Fujita-Gakuen University School of Medicine, Kutsukake-cho, Toyoake 470-11, Japan.

MATERIALS AND METHODS

Male Wistar rats (250-300 g) were used. Standard laboratory chow containing 5.1% (w/w) lipid was obtained from the Oriental Yeast Co., Tokyo, Japan. All animals were maintained in a room in which the artificial lights were switched on at 7:30 a.m. and off at 7:30 p.m. for at least 7 days prior to the experiment. Except as noted, the rats were allowed access to food during the dark phase. In the fasting experiments, the animals were deprived of food beginning at 7:30 a.m. (0h). Diabetes was induced by a single intravenous injection of streptozotocin (Sigma. Co., St. Louis, Missouri) amounting to 65 mg/kg body weight. Diabetic animals were used 3 to 4 days after the injection, by which time plasma glucose concentrations exceeded 350 mg/dl. Some diabetic animals were treated by daily subcutaneous injection of lente insulin (Novo Laboratory, Copenhagen, Denmark), 6 units per animal at 5 p.m. for 4 days. The insulin injection of the 4th day was omitted from a part of the treated rats (insulin-withdrawn group). Insulin-treated animals were fed on the diet ad libitum in order to prevent hypoglycemia. All animals were killed by decapitation. The blood was collected in ice-chilled tubes

for the determination of plasma insulin and metabolites. Plasma immunoreactive insulin (IRI) was measured by a double antibody radio-immunoassay (11). Metabolite concentrations were determined as follows: glucose by the method of Stevens (12); triglyceride by the method of Fletcher (13) using Triglyceride-Test (Wako Pure Chemical Industries, Osaka, Japan); free fatty acids (FFA) by Wako's NEFA C-Test using acyl-CoA oxidase (14). Total ketone bodies were calculated by the summation of acetoacetate and β-hydroxybutyrate determined as described by Williamson et al. (15). Lipase activity was measured in liver, heart, diaphragm (red muscle), psoas minor (white muscle) and in adipose tissue (epididymal fat body). Liver slices were prepared with a slicer. Other tissues were cut into about 20 mg pieces. After rinsing in approximately 100 ml of chilled Krebs-Henseleit buffer, pH 7.4, 200 mg of tissue was weighed. All tissues were homogenized in Krebs-Henseleit buffer containing 5 units of heparin per ml with an Ultra-Turrax (Janke u. Kunkel KG, West Germany) for 30 sec. Liver, heart and diaphragm were homogenized at a tissue concentration of 40 mg wet weight per ml of homogenate; psoas minor and adipose tissue were at 70 mg wet weight per ml of homogenate. The homogenates were filtered through 4 layers of gauze to remove debris, and the resulting debris-free homogenates were assayed for lipase activity. The suspension of adipose tissue was centrifuged, the fat layer was removed and the infranatant layer was used as the enzyme source. Lipase activity was determined by the method of Krauss et al. (4). The triolein emulsion used as substrate was prepared by sonicating the following components: 1) tri-[1-^{14}C]-oleate (99.7 mCi/mmole, New England Nuclear, Boston, Massachusetts), 127 nmoles; 2) unlabeled triolein (Sigma, St. Louis, Missouri), 113 μmoles; 3) FFA-free bovine serum albumin (Sigma), 250 mg; 4) Triton X-100 (Wako), 0.6 ml of a 1% aqueous solution; 5) 11.4 ml of Tris-HCl buffer, 0.194 M, pH 8.6, containing 0.15 M NaCl. These constituents were sonicated 2 times for 2 min with a 1 min interval in an ice bath with a Branson Sonifier (Branson Sonic Power Co., Danbury, Connecticut, model W-200P) at a setting of output 5. In most cases, 0.3 ml of this emulsion was mixed with an equal volume of the enzyme source and 0.06 ml of plasma obtained from rats fasted overnight. The addition of plasma was omitted from H-TGL assay. Incubation was conducted at 37 C and continued for 60 min over which time the reaction was shown to be linear. Extraction of FFA was performed by the procedure of Schotz et al. (16) and ^{14}C-labeled FFA released into the assay medium were counted, using the Aloka liquid scintillation system LSC-751. It was equipped with automatic external standardization to measure quenching (Aloka, Tokyo, Japan). Results were expressed as μmoles of FFA release/h/g tissue. All the experimental data were expressed as means ± SEM. The statistical significance of the difference was analyzed by Student's t test.

RESULTS

Properties of Tissue Specific Lipases

Characteristics of each tissue lipase obtained from normal fed rats are shown in Table 1. In the absence of serum, enzyme activities of adipose tissue, heart, diaphragm and psoas minor were largely inhibited, whereas that of liver was not affected. Preincubation with 1 M NaCl produced a marked reduction of enzyme activity in the tissues other than liver. The results indicate that the enzyme prepared from liver was identical to the properties of H-TGL, and other enzymes were identical to those of LPL (5). Essentially similar results were obtained with enzymes from STZ diabetic rats.

Effects of Fasting on Tissue Specific Lipase Activities in Normal Rats

As shown in Fig. 1, in normal rats fasted continuously for 48 hr, the patterns of variation of lipase activities determined at 12 hr intervals were shown to be classified into two types. The first pattern, showing gradual decrease of the enzyme activity during the time course, was seen in liver, adipose tissue and psoas minor. The second one was observed in heart and diaphragm, whose enzyme activities were not reduced by fasting. Especially the lipase activity of heart showed statistically significant increase between 12 hr and 36 hr, although it returned to normal after 48 hr fasting. In some cases, the lipase activities released by heparin (5U/ml) from the tissues during 45 min incubation were measured as described by LaRosa et al. (3). Heparin-released lipase activities (mean ± SEM, N=5) obtained for fed and 24 hr starved rats were as follows: liver, 8.2 ± 0.5 and 4.0 ± 0.9 μmoles/h/g tissue, respectively; adipose tissue, 2.9 ± 0.3 and 0.3 ± 0.1 μmoles/h/g tissue, respectively; heart, 1.7 ± 0.2 and 3.1 ± 0.1 μmoles/h/g tissue, respectively. The alteration of heparin-released TGL activities resembled that of tissue homogenates. No significant heparin releasable lipase activity was observed either in diaphragm or in psoas minor in the present study. The rats deprived of food over a 48 hr period showed the expected decline of

TABLE 1

Characteristics of the Lipase in Different Tissues[a]

Condition of assay	Enzyme activity (relative to complete system)				
	Liver	Adipose tissue	Psoas minor	Heart	Diaphragm
Complete system	100%	100%	100%	100%	100%
No serum	95.2 ± 1.4	7.7 ± 4.3	25.7 ± 1.8	16.8 ± 3.3	14.5 ± 2.9
NaCl (1 M)	82.4 ± 2.0	5.5 ± 1.7	11.6 ± 5.8	6.0 ± 2.5	15.7 ± 6.6

[a]Effect of 1 M NaCl was studied by the preincubation of enzyme with 1 M NaCl for 10 min at 37 C prior to 60 min incubation. Results are expressed as means ± SEM for four determinations.

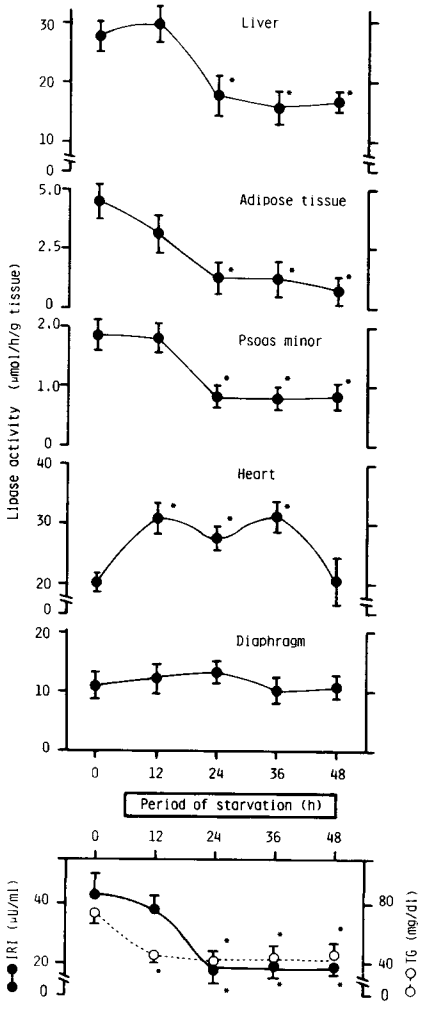

FIG. 1. Effect of fasting on lipase activity of different tissues and plasma insulin and triglyceride levels. Rats were deprived of food beginning at 7:30 a.m. (0h). Five pairs of rats were killed at each time interval. Each point represents the mean ± SEM. *:P<0.05, compared with fed.

plasma IRI and triglyceride levels (the bottom of Fig. 1). On the other hand, levels of plasma FFA and total ketone bodies were raised by fasting. The concentrations (mean ± SEM, N=6) obtained for fed and 24 hr starved rats were as follows: FFA, 0.23 ± 0.04 and 0.60 ± 0.15 mM, respectively; total ketone bodies, 0.08 ± 0.04 and 1.12 ± 0.40 mM, respectively.

Effects of STZ Diabetes on Tissue Specific Lipase Activities

The concentrations of plasma metabolites for insulin-treated rats are shown in Table 2. In terms of achieving normoglycemia and for reduced plasma FFA, triglyceride and ketone bodies, the administered insulin dose was estimated to be the optimal dose. Overnight withdrawal of insulin from the treated rats produced a marked rise in plasma glucose, triglyceride, FFA and total ketone bodies (Table 2). The plasma IRI level was reduced markedly. Withdrawal of insulin induced a significant fall in both liver and adipose tissue lipase activities and an increase in those of the heart and diaphragm (Table 2). Psoas minor lipase activity did not change significantly. Alteration of enzyme activities caused by withdrawal of insulin from diabetic rats was very similar to that caused by short-term (24 hr) fasting in normal rats.

Effects of Fasting on Tissue Specific Lipase Activities of STZ Diabetic Rats

Plasma IRI, glucose, FFA and total ketone bodies of diabetic rats did not change significantly as a result of 24 hr fasting (Table 3). However, the triglyceride level was very high in the fed state, and it was decreased significantly by fasting. The data of lipase activity in Table 3 clearly indicate that the effects of fasting on the tissue specific lipase activities were lost in the diabetic animals.

DISCUSSION

The experiments reported in this study have

TABLE 2

Effect of Insulin-withdrawal from Insulin-treated Diabetic Rats on Plasma Insulin and Metabolites and Lipase Activity of Different Tissues[a]

	Insulin-treated[b]	Insulin-withdrawn[c]
Plasma		
IRI (μU/ml)	220 ± 41	23 ± 3*
Glucose (mg/dl)	110 ± 20	508 ± 17*
TG (mg/dl)	37 ± 4	88 ± 5*
FFA (mM)	0.15 ± 0.01	0.72 ± 0.07*
Total ketone bodies (mM)	0.15 ± 0.01	2.49 ± 0.89*
Lipase activity (μmol/h/g tissue)		
Liver	28.2 ± 1.7	21.7 ± 0.5*
Adipose tussue	10.6 ± 1.3	1.6 ± 0.3*
Psoas minor	1.3 ± 0.1	1.3 ± 0.2
Heart	28.4 ± 2.2	50.4 ± 2.7*
Diaphragm	5.0 ± 0.4	8.1 ± 0.9*

[a]Rats were fed on the diet ad libitum up to the time of killing. Five pairs of rats were killed at 8 a.m. Results are expressed as means ± SEM. Values which are significantly different from insulin-treated rats are indicated by *:$P<0.05$.

[b]Diabetic rats were treated by daily subcutaneous injection of insulin, 6 units per animal at 5 p.m. for 4 days.

[c]Insulin was omitted in the previous evening.

TABLE 3

Effect of Fasting on Plasma Insulin and Metabolites and Lipase Activity of Different Tissues in Streptozotocin-diabetic Rats[a]

	Fed[b]	Fasted[c]
Plasma		
IRI (μU/ml)	9 ± 1	9 ± 1
Glucose (mg/dl)	431 ± 16	373 ± 22
TG (mg/dl)	753 ± 115	146 ± 37*
FFA (mM)	1.03 ± 0.15	0.80 ± 0.07
Total ketone bodies (mM)	5.61 ± 1.20	4.24 ± 1.00
Lipase activity (μmol/h/g tissue)		
Liver	16.3 ± 1.2	15.5 ± 0.6
Adipose tussue	0.37 ± 0.04	0.59 ± 0.14
Psoas minor	0.65 ± 0.10	0.57 ± 0.05
Heart	16.0 ± 3.9	18.0 ± 4.1
Diaphragm	4.7 ± 0.7	4.0 ± 0.5

[a]Six pairs of rats were killed at 7:30 a.m. Results are expressed as means ± SEM. Values which are significantly different from fed diabetic rats are indicated by *:$P<0.05$.

[b]Rats were allowed access to food during the dark phase.

[c]Rats were deprived of food for 24 hr beginning at 7:30 a.m.

shown that H-TGL activity and LPL activities of adipose tissue and psoas minor are reduced by starvation of normal rats, while LPL activities of heart and diaphragm are not decreased, especially the former, which significantly increased during the early stages of fasting. The observed changes in respective tissue lipase activities are consistent with the previous findings of others that fasting of normal rats decreased lipase activities of liver (17,18) and adipose tissue (17,19-21), while it increased those of heart (22-24) and diaphragm (22,24). A new finding in the present study is that withdrawal of insulin from insulin-treated STZ diabetic rats produced a change in the activities of tissue specific lipase very similar to that by fasted normal rats. There was a decrease of lipase activities in both liver and adipose tissue and an increase of that in both heart and diaphragm (see Table 2). Furthermore, when insulin-deficient diabetic animals were fasted for 24 hr, no alteration of lipase activities was observed (Table 3). Chen et al. (25) also indicated that insulin-deficient rats were incapable of increasing adipose tissue LPL activity in response to eating. Among earlier studies,

only Elkeles et al. (17) investigated the effects of streptozotocin diabetes both on LPL activities of different tissues (adipose tissue and heart) and H-TGL activity of liver. They also demonstrated that H-TGL behaved like adipose tissue LPL in rats from which insulin had been withdrawn. In the present study, determination of plasma IRI level indicated that changes of the enzyme activities, especially of liver, adipose tissue and heart, coincided with those of plasma IRI levels (see Fig. 1 and Table 2). This strongly suggests that insulin is the hormone involved in the regulation of the tissue specific lipase system. Hormonal regulation of heart LPL activity is still controversial. Borensztajn et al. (23) postulated that the heart lipase activity might be regulated by the mechanism different from insulin. Nevertheless, in later studies (26), they found no correlation between plasma glucagon level and heart lipase activity. The results of the present study showing increased activity of heart LPL by both fasting and diabetes are in line with the results reported by Kessler (27), in which he pointed out the possible regulation of heart lipase by insulin. Further investigation is required to clarify the hormonal regulation of heart lipase.

The evidence that the tissue lipase activity is an important determinant of the ability of tissues to remove plasma triglyceride from the circulation has been reviewed (2,28). Thus, it is probable that the changes of enzyme activities among the tissues under the hormonal and nutritional environment might determine the direction of plasma triglyceride to certain tissues. So far, the role of the LPL system in governing the uptake of plasma triglyceride fatty acids (TGFA) has been somewhat elucidated (21,22,24,29,30), while the fuction of H-TGL remains obscure. In the present study it was clearly indicated that the lipase activities of liver and adipose tissue and that of the heart are reciprocally changed, not only by 24 hr starvation of normal rats, but also by withdrawal of insulin from streptozotocin diabetic animals. Our data also show that lipase activity of the diaphragm is greater than that of psoas minor, which uses mainly carbohydrate as fuel (see Fig. 1 and Table 2), and that the calculated ratio of the activity of diaphragm LPL to that of psoas minor LPL was increased from 6.1 to 15.2 by 24 hr starvation of normal rats and also increased from 3.8 to 6.2 by withdrawal of insulin from diabetic animals. Therefore, it is strongly suggested that when carbohydrate supply to the tissues is restricted, either by fasting or by diabetes, the lipase system serves to direct TGFA away from liver, adipose tissue and white muscles (e.g., psoas minor) to the tissues such as heart and red skeletal muscles (e.g., diaphragm) as an energy source. Knauer et al. (31) pointed out the inverse relationship between H-TGL activity and plasma FFA level. Our data from fasting and insulin-withdrawal experiments show that H-TGL activity is decreased when plasma FFA and ketone bodies are high. It is well known the liver becomes the organ producing ketone bodies for the peripheral tissues under the condition of fasting or diabetes (32). Therefore, the present study strongly suggests that the decreased H-TGL activity, whether caused by fasting or diabetes, might serve to direct TGFA away from the uptake by the liver to the peripheral tissues including heart and red skeletal muscles and that consequently the latter tissues can utilize TGFA as well as FFA and ketone bodies as a source of energy under these circumstances.

What is a contributing factor to the elevation of plasma triglyceride in diabetic animals is not yet clear. According to Reaven et al. (33), the development of hypertriglyceridemia in streptozotocin diabetic rats is possibly caused by a defect in the removal of triglyceride-rich lipoproteins from plasma. Two lines of evidence obtained in the present work suggest the involvement of lipases especially of liver and adipose tissue. First, our data showed that withdrawal of insulin from the insulin-treated diabetic rats caused a rise in plasma triglyceride level with simultaneous reduction in lipase activities of liver and adipose tissue (Table 2). Secondly, it was observed that the hypertriglyceridemia was greatly exaggerated by feeding of diabetic animals, under which condition these two lipase activities remained low (Table 3). It is therefore assumed that the reduced lipase activities of liver and adipose tissue might decrease the amount of removal of triglyceride from the circulation and at least partially explain the cause of hypertriglyceridemia. However, which lipase is most responsible for the development of hypertriglyceridemia remains to be elucidated.

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REFERENCES

1. Carlson, L.A., and Böttiger, L.E. (1972) Lancet 1, 865-868.
2. Robinson, D.S. (1970) in Comprehensive Biochemistry (Florkin, M. and Stotz, E.H., eds.)

vol. 18, pp. 51-116, Elsevier, Amsterdam.
3. LaRosa, J.C.; Levy, R.I.; Windmueller, H.G.; and Fredrickson, D.S. (1972) J. Lipid Res. 13, 356-363.
4. Krauss, R.M.; Windmueller, H.G.; Levy, R.I.; and Fredrickson, D.S. (1973) J. Lipid Res. 14, 286-295.
5. Nilsson-Ehle, P.; Garfinkel, A.S.; and Schotz, M.C. (1980) in Ann. Rev. Bioch. (Snell, E.E., Boyer, P.D., Meister, A. and Richardson, C.C., eds.) vol. 49, pp. 667-693, Annual Reviews Inc., California.
6. Tan, M.H. (1978) Can. Med. Assoc. J. 18, 675-680.
7. Jansen, H., and Hülsmann, W.C. (1980) TIBS 5, 265-268.
8. Jansen, H., and Hülsmann, W.C. (1975) Biochim. Biophys. Acta 398, 337-346.
9. Nakai, T.; Yamada, S.; Tamai, T.; Kobayashi, T.; Hayashi, T.; and Takeda, R. (1979) Metabolism 28, 30-40.
10. Applebaum, D.M.; Goldbert, A.P.; Pykälistö, O.J: Brunzell, J.D.; and Hazzard, W.R. (1977) J. Clin. Invest. 59, 601-608.
11. Morgan, C.R., and Lazarow, A. (1963) Diabetes 12, 115-126.
12. Stevens, J.F. (1971) Clin. Chim. Acta 32, 199-201.
13. Fletcher, M.J. (1968) Clin. Chim. Acta 22, 393-397.
14. Shimizu, S.; Yasui, K.; Tani, Y.; and Yamada, H. (1979) Biochem. Biophys. Res. Commun. 91. 108-113.
15. Williamson, D.H.; Mallanby, J.; and Krebs, H.A. (1962) Biochem. J. 82, 90-96.
16. Schotz, M.C.; Garfinkel, A.S.; Huebotter, R.J.; and Stewart, J.E. (1970) J. Lipid Res. 11, 68-69.
17. Elkeles, R.S., and Hambley, J. (1977) Diabetes 26, 58-60.
18. Nomura, T.; Iguchi, A.; Matsunaga, H.; and Sakamoto, N. (1982) Lipids 18, 573-575.
19. Wing, D.R., and Robinson, D.S. (1968) Biochem. J. 106, 667-676.
20. Cryer, A.; Riley, S.E.; Williams, E.R.; and Robinson, D.S. (1976) Clin. Sci. Mol. Med. 50, 213-221.
21. Tan, M.H.; Sata, T.; and Havel, R.J. (1977) J. Lipid Res. 18, 363-370.
22. Borensztajn, J.; Otway, S.; and Robinson, D.S. (1970) J. Lipid Res. 11, 102-110.
23. Borensztajn, J.; Samols, D.R.; and Rubenstein, A.H. (1972) Am. J. Physiol. 223, 1271-1275.
24. Linder, C.; Chernick, S.S.; Fleck, T.R.; and Scow, R.O. (1976) Am. J. Physiol. 231, 860-864.
25. Chen, Y.-D.I.; Risser, T.R.; Cully, M.; and Reaven, G.M. (1979) Diabetes 28, 893-898.
26. Kotlar, T.J., and Borensztajn, J. (1977) Am. J. Physiol. 233,E316-319.
27. Kessler, J.I. (1963) J. Clin. Invest. 42, 362-367.
28. Scow, R.O.; Blanchette-Mackie, E.J.; and Smith, L.C. (1976) Circ. Res. 39, 149-162.
29. Hamosh, M.; Clary, T.R.; Chernick, S.S.; and Scow, R.O. (1970) Biochim. Biophys. Acta 210, 473-482.
30. Chan, C.P., and Stern, J.S. (1982) Am. J. Physiol. 242, E455-450.
31. Knauer, T.E.; Woods, J.A.; Lamb, R.G.; and Fallon, H.J. (1982) J. Lipid Res. 23, 631-637.
32. Newsholme, E.A., and Start, C. (1976) Regulation in Metabolism, pp. 293-323, John Willey and Sons, New York.
33. Reaven, G.M., and Greenfield, M.S. (1981) Diabetes 30 (Suppl. 2), 66-75.

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Studies on Peroxidized Lipids. V. Formation and Characterization of 1,4-Dihydropyridine-3,5-Dicarbaldehydes as Model of Fluorescent Components in Lipofuscin

K. KIKUGAWA* and Y. IDO, *Tokyo College of Pharmacy, 1432-1 Horinouchi, Hachioji, Tokyo 192-03, Japan*

ABSTRACT

We investigated fluorescence properties of 1,4-dihydropyridine-3,5-dicarbaldehydes and their formation in mild reaction of primary amines and malonaldehyde, in order to clarify the role of malonaldehyde in the formation of fluorescent components of lipofuscin. The compounds exhibited fluorescence with excitation maxima at 375-405 nm and emission maxima at 435-465 nm, which was similar to those of lipofuscin and the fluorescent substances derived from the reaction of oxidized fatty acids with primary amines. Fluorescence of the compounds was greatly affected in acidic medium and little influenced in alkaline medium or by the metal chelator. The compounds lost fluorescence by treatment with sodium borohydride. They were inert to thiobarbituric acid reaction. Some of the fluorescence properties of the compounds were different from those of lipofuscin and the related fluorescent substances. Mild reaction of methylamine with pure malonaldehyde gave a single fluorescent compound, 1,4-dimethyl-1,4-dihydropyridine-3,5-dicarbaldehyde (Ia), and the reaction with the acid hydrolysate of tetramethoxypropane gave Ia and 1-methyl-4-(dimethoxyethyl)-1,4-dihydropyridine-3,5-dicarbaldehyde (IIa), the latter being produced from the impurity in the hydrolysate. These reactions produced a non-fluorescent Schiff base, a 1:1-adduct of methylamine and malonaldehyde (IIIa), as a major product. It looks unlikely that malonaldehyde is the only product of lipid oxidation that produces fluorescent components in lipofuscin complex.

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INTRODUCTION

Polyunsaturated fatty acids are oxidized to produce a mixture of several products including malonaldehyde (1). This sequence of reactions has been regarded generally as being involved in cell damage, and in the formation of lipofuscin with characteristic fluorescence (2-4). The structure of the fluorescent components found in lipofuscin complex had been assumed to be the conjugated Schiff base between malonaldehyde and primary amino groups of proteins or phospholipids, on the basis of in vitro experiments described by Chio and Tappel (5-7). They treated primary amines with malonaldehyde, prepared by acid hydrolysis of tetramethoxypropane (TMP), and obtained amorphous conjugated Schiff bases (N,N′-disubstituted 1-amino-3-iminopropenes; RNHCH=CHCH=NR) (5) with fluorescence maxima similar to those of lipofuscin complex (4). Previously we isolated another type of fluorescent compound, 1,4-dihydropyridine-3,5-dicarbaldehydes (I and II), in crystalline form from the reaction of primary amines with the TMP hydrolysate at neutral pH, and the structure of these compounds was unambiguously established (8,9).

In this paper, we investigated the fluorescence properties of these compounds to compare them with those of lipofuscin complex and the fluorescent substances derived from the reaction of primary amines and oxidized lipid, and their formation in mild reaction of primary amines and malonaldehyde. We also discussed the role of malonaldehyde on the formation of the fluorescent components in lipofuscin complex.

*To whom correspondence should be addressed.

EXPERIMENTAL PROCEDURES

Materials

Tetramethoxypropane (TMP) was the product of Tokyo Kasei Kogyo Company, Ltd., Tokyo. Europium-tris(2,2,6,6-tetramethyl-3,5-heptanedionate, [$Eu(thd)_3$] was the product of Fluka AG, Chemische Fabrik, Switzerland. 1,4-Dimethyl-1,4-dihydropyridine-3,5-dicarbaldehyde (Ia), 1-n-hexyl-4-methyl-1,4-dihydropyridine-3,5-dicarbaldehyde (Ib), 3,5-diformyl-4-methyl-1,4-dihydropyridine-1-acetic acid ethyl ester (Ic) and 1-methyl-4-(dimethoxyethyl)-1,4-dihydropyridine-3,5-dicarbaldehyde (IIa) were prepared by reaction of the corresponding amines with TMP hydrolysate at pH 7 and 37 C according to the methods described elsewhere (8,9). 3,5-Diformyl-4-methyl-1,4-dihydropyridine-1-acetic acid (Id) was prepared by treatment of Ic with mild alkali (9). N-(2-formylvinyl)-glycine (IIId), the 1:1 Schiff base between

glycine and malonaldehyde, was prepared as described (9,10).

Analytical Methods

Ultraviolet absorption was determined by use of a UV-200S Shimadzu double beam spectrophotometer. Nuclear magnetic resonance (NMR) spectra were obtained using a JEOL PS-100 instrument. Fluorescence was determined with a Hitachi MPF-4 fluorescence spectrophotometer, and the relative intensity was expressed relative to 0.1 μM quinine sulfate in 0.1 N sulfuric acid with excitation at 352 nm and emission at 448 nm. Buffers used for fluorescence measurements were 0.1 M glycine-0.1 N HCl (pH 2 and 3), 0.1 M acetate (pH 4 and 5), 0.1 M phosphate (pH 6 and 7), 0.1 M Tris-HCl (pH 8 and 9) and 0.4 M glycine-0.1 N NaOH (pH 10 and 11). Thin layer chromatography (TLC) was performed on a Silica gel $70F_{254}$ plate-Wako with a solvent system, ethyl acetate: ethanol (8:2).

The fluorescent products in reaction mixtures were analyzed by high performance liquid chromatography (HPLC) on a Shimadzu LC-2 liquid chromatograph with a stainless steel column (4.6 mm i.d. x 25 cm) of Zorbax ODS, eluting with a mixture of methanol-water (1:1) at a flow rate of 0.7 ml/min. The peaks were detected by fluorescence at 460 nm with excitation at 400 nm with a Shimadzu RF-530 fluorescence spectromonitor. Authentic compounds, Ia and IIa, eluted at retention times of 6.8 and 6.0 min, respectively. The peak heights of Ia and IIa showed a linear relationship with their concentrations.

Thiobarbituric Acid Test

The thiobarbituric acid (TBA) test was performed according to the previously described methods (11,12) with modifications. In Method 1 a mixture of 0.10 ml sample solution, 2.9 ml 0.5% trichloroacetic acid (TCA) and 1.0 ml 0.5% TBA was heated at 60 C for 90 min. After cooling the mixture, the absorption spectrum was monitored between 400 and 600 nm. In Method 2 a mixture of 0.10 ml sample solution, 3.0 ml glacial acetic acid and 3.0 ml 0.5% TBA was heated at 100 C for 20 min to measure absorbance.

In both methods, each mixture contained more than 35 μmol TBA and less than 2 μmol test compound, and the standard samples (pure malonaldehyde and TMP) produced red pigment with equal absorbance at 532 nm.

TMP Hydrolysate

TMP hydrolysate was prepared by two methods. For Preparation A, 1.64 g (10 mmols) of TMP was mixed with 0.90 ml 1.0 N HCl and warmed at 40 C until miscible, and made up to 10 ml with water. The acidic solution was incubated at 37 C for 1 hour before use (5,13,14). This solution contained no TMP when estimated by gas chromatography (13). For Preparation B, TMP was similarly treated with concentrated HCl instead of 1.0 N HCl.

Malonaldehyde Sodium Salt (MDA·Na)

MDA·Na was prepared according to the method of Marnett and Tuttle (15) with slight modifications. Thus, 8.2 g (50 mmols) of TMP was stirred with 33 ml Dowex 50W-X4 (H^+) in 100 ml of water at room temperature for 30 min. The resin was removed by filtration, and the filtrate was adjusted at pH 7-8 by addition of 5 N NaOH. The filtrate was extracted with an equal volume of ethyl acetate three times, the aqueous layer was evaporated in vacuo below 30 C and the residue was crystallized by addition of acetone. The crystalline MDA·Na was collected by filtration (1.35 g) and recrystallized from water-acetone. NMR (D_2O) δ 8.65 ppm (d), 5.30 (t), 4.72 (HOD). The spectrum coincided with that of MDA·Na reported by Marnett and Tuttle (15). The preparation contained about 3 mols of water by NMR analysis.

Reaction of Methylamine with TMP Hydrolysate and MDA·Na

Mixtures of (*1*) 200 mM methylamine and 50 mM TMP hydrolysate[A] (the ratio of the reactants, 4:1); (*2*) 50 mM methylamine and 100 mM TMP hydrolysate[A] (1:2); (*3*) 200 mM methylamine and 100 mM TMP hydrolysate[A] (4:2); (*4*) 50 mM methylamine and 100 mM TMP hydrolysate[B] (1:2), (*5*) 200 mM methylamine and 100 mM TMP hydrolysate[B] (4:2), or (*6*) 50 mM methylamine and 100 mM MDA·Na (1:2), in phosphate buffer (pH 7), were incubated at 37 C during the periods indicated. The fluorescence spectrum of the reaction mixture was measured after dilution with 0.1 M phosphate buffer (pH 7). TLC and HPLC of the reaction mixture were carried out to estimate the fluorescent products. Chloroform-extractable products were estimated as follows. The reaction mixture (2 ml) was mixed with 2.0 g sodium chloride and 5 ml 1 N NaOH, and extracted with 20 ml chloroform. The extracts were evaporated and dissolved in 10 ml water, and ultraviolet absorption and the fluorescence spectra of the solution were measured in 0.1 N HCl and in 0.1 M phosphate buffer (pH 7), respectively.

RESULTS

Spectral Characterization of 1,4-Dihydropyridine-3,5-Dicarbaldehydes

Ultraviolet absorption spectra of 1,4-dihydropyridine-3,5-dicarbaldehydes (I and II) were measured under identical conditions (Fig. 1 and Table 1). Compound Ia showed a characteristic absorption spectrum with maxima at 238, 264 and 401 nm at pH 7 and the spectrum was not significantly altered in 0.1 N HCl and 0.1 N NaOH, indicating that it had no dissociation of protons between pH 1 and 13. Compounds Ia, b,c,d were stable after incubation at 37 C for 16 hours in phosphate buffer (pH 7), 0.1 N HCl or 0.1 N NaOH. However, compound IIa was unstable and degraded in 0.1 N HCl at 37 C.

Fluorescence spectra of compounds I and II in various solvents are shown in Fig. 2 and Table 2. Compound Ia revealed an excitation maximum at 403 nm and an emission maximum at 462 nm in phosphate buffer (pH 7) with higher relative molar intensity than quinine sulfate. Excitation and emission maxima of compounds Ib,c and IIa in phosphate buffer (pH 7) were very close to those of compound Ia, but those of Ic were shifted to shorter wavelength. When the spectra of Ia,b,c and IIa were measured in methanol, ethanol,

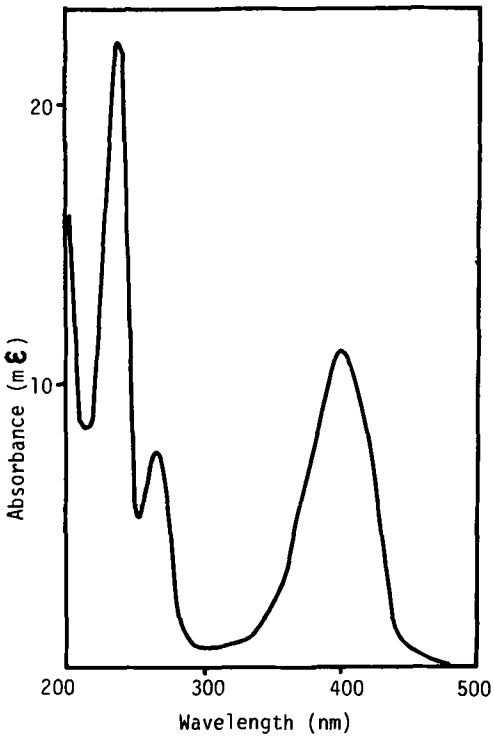


FIG. 1. Absorption spectrum of Ia in 0.1 M phosphate buffer (pH 7.0).

TABLE 1

Ultraviolet Absorption Maxima of I and II[a]

	UV: nm (mε)		
	λ_{max}(0.1 M phosphate, pH 7)	λ_{max}(0.1 N HCl)	λ_{max}(0.1 N NaOH)
Ia[b]	238 (22) 264 (7.6) 401 (11.3)	236 (22.5) 265 (7.3) 401 (11.3)	236 (23) 264 (8.1) 401 (11.1)
Ib	238 (25.4) 265 (9.1) 402 (12.2)	238 (25.6) 264 (8.8) 402 (12)	238 (25.1) 264 (9.8) 402 (12)
Ic	236 (22.4) 264 (9.5) 384 (10.7)	236 (22.4) 264 (9.5) 384 (10.7)	
Id	238 (24.6) 265 (9.7) 394 (11.8)	236 (23.5) 263 (9.6) 384 (11.3)	238 (25.3) 265 (10.3) 394 (11.8)
IIa	236 (21.7) 264 (7.2) 394 (10.6)	236 (22.4) 264 (7.3) 396 (10.8)	236 (21.7) 264 (9.1) 394 (10.3)

[a]All the spectra were unchanged by incubation at 37 C for 16 hours, except for the spectrum of IIa in 0.1 N HCl, which lost 96% absorbance at 396 nm.
[b]See Fig. 1.

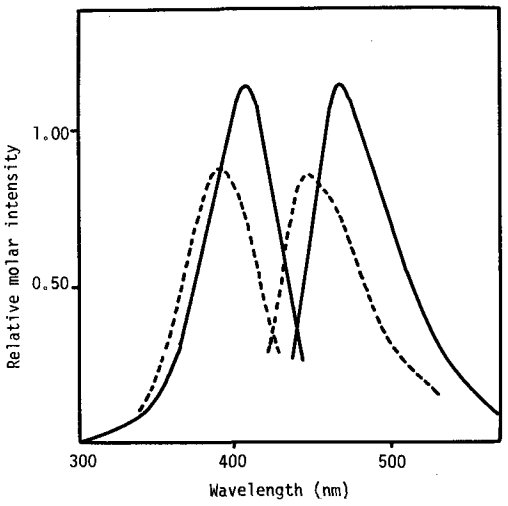


FIG. 2. Fluorescence spectrum of Ia in 0.1 M phosphate buffer (pH 7.0) (——) and in chloroform (----).

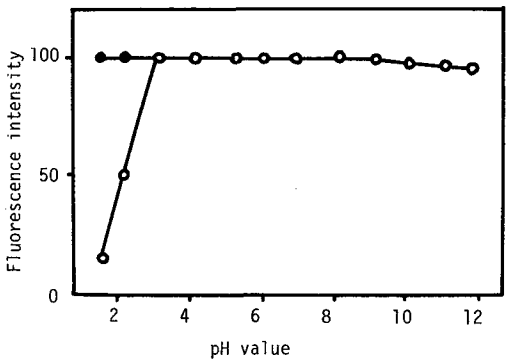


FIG. 3. Fluorescence intensity of Ia in solutions with different pH values. Open circles: intensity at the pH value indicated. Closed circles: intensity after neutralization.

isopropanol and chloroform, both excitation and emission maxima shifted to shorter wavelength. The shifts in wavelength were the largest in chloroform. The relative molar intensities of these compounds in methanol, ethanol and isopropanol were different with each compound, and their intensity in chloroform decreased markedly, probably due to the quenching effect of this solvent.

Fluorescence spectra of Ia were measured in aqueous solutions at different pH values. Excitation and emission maxima were not altered between pH 1 and 12, but the intensity was markedly decreased below pH 4 (Fig. 3). This decrease could not be due to the changes in electronic structure nor to degradation, but rather to the solvent effect, since subsequent treatment with alkali restored the fluorescence

TABLE 2

Fluorescence Spectra of I and II[a]

Solvent	Ia[b]			Ib			Ic			Id			IIa		
	Ex	Em	RMI	Ex	Em	RMI	Ex	Em	RMI	Ex	Em	RMI	Ex	Em	RMI
0.1 M phosphate (pH 7.0)	403	462	1.27	402	461	1.43	390	449	0.89	399	458	1.42	400	461	0.84
MeOH	392	451	1.18	395	452	1.14	386	440	0.66	395	450	1.38	398	455	0.53
EtOH	397	452	1.23	395	452	1.15	386	439	0.70	397	453	1.38	398	455	0.59
i-BuOH	397	452	1.23	395	450	1.43	386	439	0.73	398	454	1.59	400	453	0.69
$CHCl_3$	390	446	0.93	391	443	1.03	378	445	0.36	380	440	0.29	393	450	0.38

[a]Ex: excitation maximum (nm). Em: emission maximum (nm). Relative molar intensity (RMI) of every compound was expressed relative to quinine sulfate.
[b]See Fig. 2.

TABLE 3

Fluorescence Intensity of I and II under Various Conditions

	Treatment			
	0.1 N HCl[a,b]	0.1 N NaOH[a]	2 mM $NaBH_4$ [a,c]	40 μM $Eu(thd)_3$/MeOH[d]
Ia[e]	13	95	3	100
Ib	13	100	7	100
Ic	19	—	9	100
Id	12	98	6	100
IIa	19	85	4	—

[a]Fluorescence intensity was expressed as percentage of that obtained in 0.1 M phosphate buffer (pH 7.0).
[b]The intensity was increased to about 100 by neutralization.
[c]1.25 μM compound was treated with 2 mM $NaBH_4$ at room temperature for 10 min and diluted to 0.1 μM concentration with 0.1 M phosphate buffer (pH 7.0).
[d]Fluorescence intensity was expressed as percentage of that obtained in MeOH.
[e]See Fig. 3.

intensity. Other 1,4-dihydropyridine-3,5-dicarbaldehydes exhibited similar decreases in fluorescence intensity in acidic conditions (Table 3). When these compounds were treated with 2 mM sodium borohydride, fluorescence intensities were reduced to less than 10% of the initial intensity (Table 3). Treatment of these compounds with the chelating agent, europium-tris(2,2,6,6-tetramethyl-3,5-heptanedionate) (7), gave no decrease in fluorescence intensity (Table 3).

TBA Reaction of 1,4-Dihydropyridine-3,5-Dicarbaldehydes

The TBA test of 1,4-dihydropyridine-3,5-dicarbaldehydes (I and II) was performed in the presence of TCA (method 1) and glacial acetic acid (method 2) (Fig. 4). While the 1:1 Schiff base (IIId) between glycine and malonaldehyde (10) showed a maximum at 532 nm similar to that of TMP or malonaldehyde with both methods, the spectrum obtained with 1,4-dihydropyridine-3,5-dicarbaldehydes (I and II) was quite different. Compounds Ia,b,c,d exhibited a maximum around 430 nm (method 1) and at 490 and 525 nm (method 2), whereas IIa was not colored with either method. Absorbance at 532 nm of IIId was about 70% of that of TMP with either method, but absorbance of I and II was less than 1% (method 1) or less than 6% (method 2) (Table 4). It was found that compounds I and II were essentially inert to TBA reaction and did not liberate malonaldehyde in acidic conditions, in contrast to the fact that the 1:1 Schiff base (IIId) readily liberated malonaldehyde in acidic conditions to produce red pigment (10,16).

Characterization of Reaction Between Methylamine and Malonaldehyde

It has been stated (8) that the reaction of

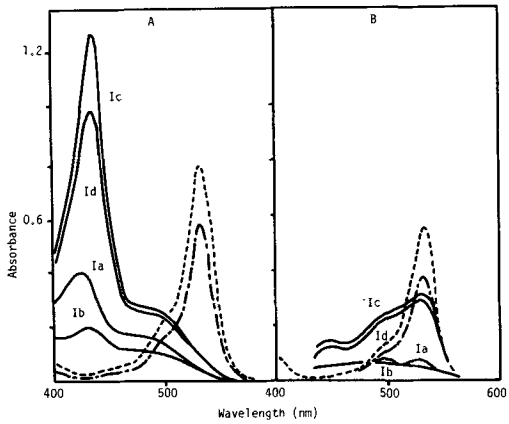


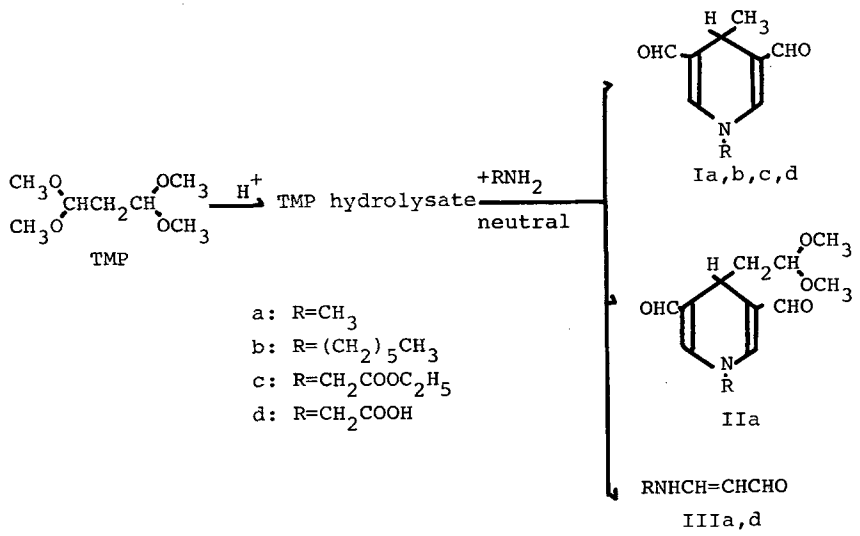
FIG. 4. Absorption spectrum of TBA reaction mixture of Ia,b,c and d. Spectrum was measured after reaction of 2 mM solution of Ia,b,c and d (——), 0.2 mM solution of TMP (-----), or 0.2 mM solution of IIId (– – –) with TBA. A: TBA Method 1, and B: TBA Method 2.

TABLE 4

Ratio of Absorbance at 532 nm in TBA Reaction

	Method 1	Method 2
TMP	1.00	1.00
Ia	0.006	0.014
Ib	0.004	0.007
Ic	0.01	0.057
Id	0.01	0.054
IIa	0.001	0.002
IIId	0.74	0.68

methylamine and TMP hydrolysate[A] under mild conditions produced the fluorescent compounds (Ia and IIa) and the 1:1 Schiff base (IIIa), whose structure was unambiguously established (Scheme I). In order to obtain



SCHEME I

further information on the characteristics of the reaction, the reaction of methylamine with several preparations of malonaldehyde was performed under several conditions, and the products were analyzed by ultraviolet spectrum, fluorescence spectrum, TLC and HPLC.

The mixture of methylamine and TMP hydrolysate[A] in a ratio of 4:1 (reaction *1* in "Experimental Procedures") was incubated for 96 hours. The reaction mixture afforded ultraviolet-absorbing and fluorescent substances which were extracted with chloroform, whereas the control without methylamine yielded no chloroform-extractable ultraviolet-absorbing or fluorescent compounds. The absorption spectrum of the chloroform extract revealed three maxima at 240, 270 and 400 nm (Fig. 5). The maxima at 240 and 400 nm may be due to Ia and IIa (Fig. 1 and Table I), and that at 270 nm to the 1:1 Schiff base (IIIa) (8). Absorbance at 270 nm reached a maximum after 24 hours, and absorbance at 400 nm and fluorescence increased more gradually during a period of 96 hours (Fig. 6). Fluorescence intensity of the reaction mixture due to Ia and IIa increased gradually during the period. Thus the reaction of malonaldehyde with large excess of methylamine provided the 1:1 Schiff (IIIa) more readily than 1,4-dihydropyridine-3,5-dicarbaldehydes Ia and IIa.

Formation of the fluorescent 1,4-dihydropyridine-3,5-dicarbaldehydes was investigated with the following mixtures: methylamine and TMP hydrolysate [A] in a ratio of 1:2 (reaction *2* in "Experimental Procedures"); methylamine and TMP hydrolysate[A] in a ratio of 4:2

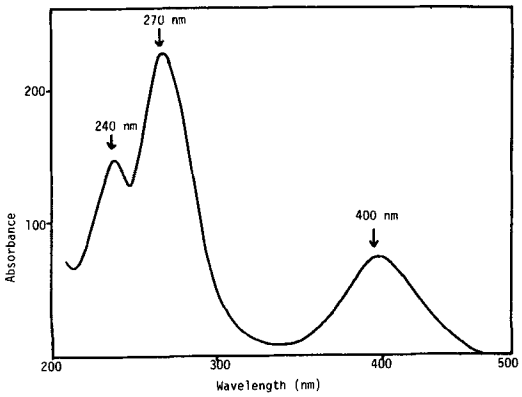


FIG. 5. Absorption spectrum of the chloroform-extractable substances in reaction mixture of 200 mM methylamine and 50 mM TMP hydrolysate [A] incubated for 96 hours. The spectrum was taken in 0.1 N HCl.

(reaction *3*); methylamine and TMP hydrolysate[B] in a ratio of 1:2 (reaction *4*); methylamine and TMP hydrolysate[B] in ratio of 4:2 (reaction *5*); and methylamine and MDA·Na in a ratio of 1:2 (reaction *6*). The reactions were performed at the different molar ratio of methylamine and malonaldehyde preparation. TMP hydrolysate[A] may contain malonaldehyde, and its intermediates or polymerized products, and TMP hydrolysate[B] may contain more polymerized products. Fluorescence spectra of every reaction mixture exhibited an excitation maximum at 405 nm and an emission maximum at 465 nm. TLC of the

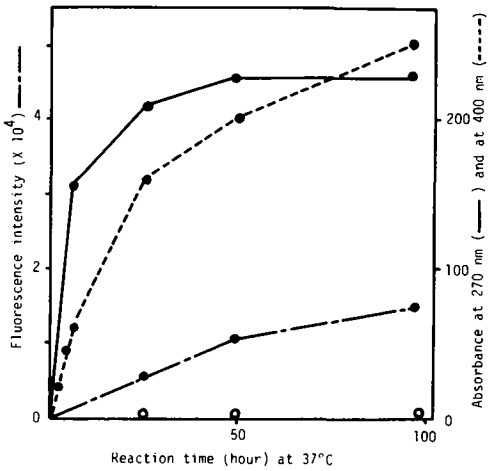


FIG. 6. Time course of increase in ultraviolet absorbing and fluorescent substances in the chloroform extracts of the reaction mixture of 200 mM methylamine and 50 mM TMP hydrolysate[A] (•) and the control without methylamine (○).

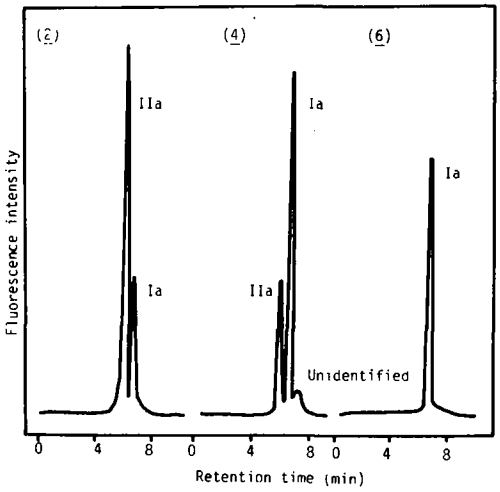


FIG. 7. HPLC of the reaction mixtures of methylamine and malonaldehyde. The mixtures of (2) 50 mM methylamine and 100 mM TMP hydrolysate[A]; (4) 50 mM methylamine and 100 mM TMP hydrolysate[B], and (6) 50 mM methylamine and 100 mM MDA·Na were incubated for 24 hours.

reaction mixtures (*2,3,4* and *5*) revealed two major fluorescent spots corresponding to Ia (Rf: 0.31) and IIa (Rf: 0.20), and the reaction mixture (*6*) gave a single fluorescent spot corresponding to Ia. On HPLC, the reaction mixtures (*2* and *3*) revealed two peaks of Ia and IIa; the reaction mixtures (*4* and *5*) two peaks of Ia and IIa with a small unidentified fluorescent peak, and the reaction mixture (*6*) a single peak of Ia (Fig. 7). Time course of the yields of

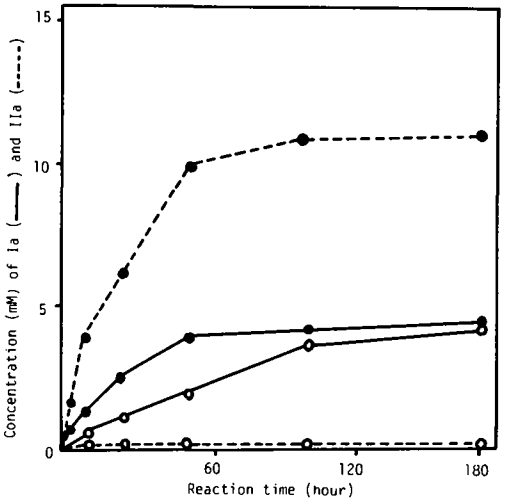


FIG. 8. Time course of formation of Ia and IIa in reaction mixtures of (2) 50 mM methylamine and 100 mM TMP hydrolysate[A] (•) and (6) 50 mM methylamine and 100 mM MDA·Na (○). Concentration of Ia and IIa was analyzed by HPLC.

Ia and IIa in reaction (*2* and *6*) were followed by HPLC (Fig. 8). HPLC of the reaction mixture with pure malonaldehyde under reaction (*6*) revealed no other fluorescent peaks than that of Ia during the test periods. The reaction mixture with TMP hydrolysate[A] (*2*) afforded fluorescent peaks corresponding to Ia and IIa, the yield of the latter being higher than that of the former. The concentration of Ia in reaction (*6*) after 180 hours was about 4 mM (8% based on methylamine), and those of Ia and IIa in reaction (*2*) were 4 mM (8%) and 12 mM (24%), respectively. Compound IIa may be derived from the reaction of methylamine and malonaldehyde and 3,3-dimethoxypropionaldehyde (15) in the TMP hydrolysate. Although data are not shown, time course and the yields of Ia and IIa in reaction (*3*) with methylamine in excess were essentially similar to those of reaction (*2*). The results indicate that Ia was the single fluorescent product derived from the reactions of methylamine and malonaldehyde under neutral conditions.

DISCUSSION

It has been suggested that aging tissue accumulates fluorescent compounds in lipofuscin complex which are derived from the in vivo reaction of oxidized lipids with proteins or phospholipids (2). Since Chio and Tappel reported that the reaction of primary amines with malonaldehyde afforded the fluorescent conjugated Schiff bases (N,N′-disubstituted 1-amino-3-iminopropenes), the 2:1-adducts of

primary amines and malonaldehyde, it has been believed that lipofuscin complex was composed of the conjugated Schiff bases (3,5). Fluorescent components in lipofuscin complex showed fluorescence spectra with excitation maxima at 340-380 nm and emission maxima at 420-470 nm (4) which were similar to those of the conjugated Schiff bases with excitation maxima at 350-396 nm and emission maxima at 450-462 nm (5). Shimasaki et al (17) demonstrated that fluorescence of lipofuscin extracted from rat testicular tissue was quenched at alkaline pH and by metal chelators, and these properties were similar to those of the conjugated Schiff bases (7).

Chio and Tappel (5) treated primary amines including amino acids with malonaldehyde, prepared by acid hydrolysis of TMP, under relatively low acidic conditions, non-physiological, to obtain the conjugated Schiff bases in amorphous state. The conjugated Schiff bases were reduced into the disubstituted amines by treatment with sodium borohydride to elucidate the structure. It has been pointed out, however, by Buttkus and Bose (16) that more rigorous assignment of the structures of the compounds may be required because the compounds were unstable and obtained in an amorphous state. Studies of the chemistry of malonaldehyde are complicated by its tendency to undergo self-condenstation reactions (18, 19), and β-methoxyacrolein and 3,3-dimethoxypropionaldehyde were also produced by acid hydrolysis of TMP (15). Nair et al. (20) demonstrated that the reaction of pure malonaldehyde with amino acids under mild conditions failed to give the conjugated Schiff bases but gave the non-fluorescent 1:1 Schiff bases. They reported that the reasons for this difference must be associated with the purity of TMP hydrolysate and the mildly acidic conditions used in their work. We made several attempts to isolate the conjugated Schiff bases from the neutral reaction of primary amines and TMP hydrolysate, but obtained 1,4-dihydropyridine-3,5-dicarbaldehydes as the major fluorescent substances whose structures were unambiguously established (8,9). Aklylamines such as methylamine and n-hexylamine and amino acids such as glycine and glycine ethyl ester produced the same kind of fluorescent compounds.

In the present investigation, we characterized the fluorescence properties of the 1,4-dihydropyridine-3,5-dicarbaldehydes (I and II) and compared them with those of the fluorescent components of lipofuscin. Compounds I and II exhibited fluorescence with excitation maxima at 390-405 nm and emission maxima at 450-465 nm in phosphate buffer (pH 7). The maxima shifted to shorter wavelength in organic solvents, and the fluorescence in chloroform exhibited excitation maxima at 375-395 nm and emission maxima at 440-450 nm. These fluorescence spectra were close to those of the fluorescent components in lipofuscin (4) and the conjugated Schiff bases (5), but the following characteristics were different. Fluorescence of compounds I and II was little influenced in alkaline medium and was greatly affected in acidic medium; the properties were greatly different from those of lipofuscin (17) and the conjugated Schiff bases (7), whose fluorescence was little influenced in acidic medium and greatly influenced in alkaline medium. Fluorescence of compounds I and II was little affected by the metal chelator, while those of lipofuscin (17) and the conjugated Schiff bases (7) were affected by the metal chelator.

In order to characterize the reaction profiles of the primary amines with malonaldehyde, reactions of methylamine with various preparations of malonaldehyde were performed under several conditions. The major product of the reaction with methylamine in excess was the non-fluorescent 1:1-Schiff base. The conjugated Schiff base composed of 2 mols of methylamine and 1 mol of malonaldehyde could be produced in reaction with methylamine in excess, but the reaction produced no such fluorescent compound. While any reactions with TMP hydrolysates [A and B] provided two fluorescent compounds Ia and IIa, the reaction with pure malonaldehyde produced a single fluorescent compound Ia. Compound Ia may be derived from malonaldehyde, but compound IIa may be derived from the mixture of malonaldehyde and 3,3-dimethoxypropionaldehyde (15), an impurity in the TMP hydrolysate. Though mechanisms of formation of Ia were not known, IIa may be formed from the reaction of 1 mol of methylamine, 2 mols of malonaldehyde and 1 mol of 3,3-dimethoxypropionaldehyde via the so-called "Hantzsch dihydropyridine synthesis" (21).

Mild reaction of primary amines with malonaldehyde produced fluorescent compounds, 1,4-dihydropyridine-3,5-dicarbaldehydes I. However, fluorescence characteristics of I were not similar to those of lipofuscin in every respect. Several studies on the formation of fluorescent pigments in reaction of primary amines with oxidized unsaturated fatty acids have appeared (22-26). In our previous paper (16), it was demonstrated that the reactions of methylamine and oxidized unsaturated fatty acids afforded many fluorescent substances with different fluorescence characteristics, and the

major products were not 1,4-dimethyl-1,4-dihydropyridine-3,5-dicarbaldehyde Ia. It is unlikely that malonaldehyde alone contributes to the formation of fluorescent components in lipofuscin or related substances.

REFERENCES

1. Gardner, W.H. (1979) J. Agric. Food Chem. 27, 220-229.
2. Miquel, J.; Oro, J.; Benesch, K.G.; and Johnson, J.E., Jr. (1977) in Free Radicals in Biology (Pryor, W.A., ed.) Vol. III, pp. 133-182, Academic Press, New York.
3. Tappel, A.L. (1980) in Free Radicals in Biology (Proyor, W.A., ed.) Vol. IV, pp. 1-47, Academic Press, New York.
4. Fletcher, B.L.; Dillard, C.J.; and Tappel, A.L. (1973) Anal. Biochem. 52, 1-9.
5. Chio, K.S., and Tappel, A.L. (1969) Biochemistry, 8, 2821-2827.
6. Chio, K.S., and Tappel, A.L. (1969) Biochemistry, 8, 2827-2832.
7. Malshet, V.G.; Tappel, A.L.; and Burns, V.M. (1974) Lipids 9, 328-332.
8. Kikugawa, K.; Maruyama, T.; Machida, Y.; and Kurechi, T. (1981) Chem. Pharm. Bull. 29, 1423-1432.
9. Kikugawa, K.; Machida, Y.; Kida, M.; and Kurechi, T. (1981) Chem. Pharm. Bull. 29, 3003-3011.
10. Crawford, D.L.; Yu, T.C.; and Sinnhuber, R.O. (1966) J. Agric. Food Chem. 14, 182-184.
11. Kwon, T.W., and Olcott, H.S. (1966) Food Science 31, 552-557.
12. Ottolenghi, A. (1959) Arch. Biochem. Biophys. 79, 355-363.
13. Kurechi, T.; Kikugawa, K.; and Ozawa, M. (1980) Food Cosmet. Toxicol. 18, 119-122.
14. Kikugawa, K.; Tsukuda, K.; and Kurechi, T. (1980) Chem. Pharm. Bull. 28, 3323-3331.
15. Marnett, L.J., and Tuttle, M.A. (1980) Cancer Res. 40, 276-282.
16. Buttkus, H., and Bose, R.J. (1972) J. Amer. Oil Chem. Soc. 49, 440-443.
17. Shimasaki, H.; Ueta, N.; and Privett, O.S. (1980) Lipids 15, 236-241.
18. Buttkus, H. (1975) J. Agric. Food Chem. 23, 823-825.
19. Gutteridge, J.M.C. (1975) Anal. Biochem. 69, 518-526.
20. Nair, V.; Vietti, D.E.; and Cooper, C.S. (1981) J. Amer. Chem. Soc. 103, 3030-3036.
21. Eisner, U., and Kuthan, J. (1972) Chem. Rev. 72, 1-42.
22. Shimasaki, H.; Ueta, N.; and Privett, O.S. (1982) Lipids 17, 878-883.
23. Shimasaki, H.; Privett, O.S.; and Hara, I. (1977) J. Amer. Oil Chem. Soc. 54, 119-123.
24. Tabata, T.; Yoden, K.; Takei, H.; and Iio, T. (1979) Yakugaku Zasshi (Tokyo) 99, 711-716.
25. Yoden, K.; Iio, T.; and Tabata, T. (1981) Yakugaku Zasshi (Tokyo) 101, 437-442.
26. Kikugawa, K.; Watanabe, S.; and Kurechi, T. (1984) Chem. Pharm Bull. 32, 631-638.

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The Effects of Hypophysectomy and Testosterone Treatment on the Composition and Metabolism of Testicular Lipids.

ZOHAIR M.H. MARZOUKI[a] and JOHN G. CONIGLIO,[b]* [a]*Presently with School of Medicine, King Abdulaziz University, P.O. Box 9029, Jeddah, Saudi Arabia,* [b]*Department of Biochemistry, Vanderbilt University, Nashville, TN 37232*

ABSTRACT

The effects of hypophysectomy and of testosterone administration on lipid composition and metabolism of rat testicular tissue have been investigated. Increased concentrations of triacylglycerols and cholesterol were observed in testes of hypophysectomized compared to control (non-hypophysectomized) rats on the eighth day posthypophysectomy. Administration of testosterone maintained the concentrations of these lipids at about normal levels. The concentration of phospholipids was not affected by the hypophysectomy. Incorporation of ^{14}C from 1-[^{14}C]linoleate into testicular lipids was determined 24 hours after intratesticular injection. In hypophysectomized compared to control rats there was more ^{14}C in C 16:0, C 20:2 and C 20:3 and less ^{14}C in C 20:4 and C 22:4 of both phospholipids and triacylglycerols. After intratesticular injection of 1-[^{14}C]eicosatrienoate there was more ^{14}C in C 16:0 and C 20:3 and less ^{14}C in C 20:4 and C 22:4 of both phospholipids and triacylglycerols of hypophysectomized compared to control rats. Intratesticular injection of 1-[^{14}C]-arachidonate resulted in less ^{14}C incorporation in C 22:4 in testes of hypophysectomized than in those of control rats. Treatment with testosterone did not affect the metabolism of any of the ^{14}C-substrates. These results indicate that the testicular desaturation of C 20:3 to arachidonate, requiring a $\Delta 5$ desaturase, is inhibited by hypophysectomy and that testosterone by itself may control the concentrations of some testicular lipid classes but not the metabolism of the polyenoic acids.
Lipids 19:609-616, 1984.

INTRODUCTION

Polyunsaturated fatty acids, particularly 22-carbon polyenoic acids, appear to have an important role in testicular tissue metabolism and in spermatogenesis in the rat (1). A three-fold increase in the concentration of docosa-4,7,10,13,16-pentaenoic acid (C 22:5) occurs in rat testes during sexual maturation (2), and most of the pentaene is associated with the germinal cells (3). The pentaene can be synthesized in the testis from dietary linoleic acid by a series of elongation and desaturation reactions (4). The predominant pathway of synthesis is: C 18:2 → C 18:3 → C 20:3 → C 20:4 → C 22:4 → C 22:5.

Essential fatty acid deficiency in the rat leads to degeneration of the testis with subsequent infertility (5,6). Similarly, hypophysectomy prevents testicular development and leads to suppressed spermatogenesis and sterility (7,8).

Although much has been learned about the pathways of the polyenoic acids in testes, little is known concerning the role of hormones in the regulation of these processes or of relationships between hormones and the lipids containing these polyenoic acids. An increase in testicular lipids in hypophysectomized rats has been reported (9,10). Luteinizing hormone and testosterone caused an increase in ^{14}C incorporation from ^{14}C linoleate into testicular polyenes of hypophysectomized rats (11). Replacement therapy following hypophysectomy did not influence the fatty acids into which ^{14}C from ^{14}C-linoleate had incorporated. The rate of catabolism of triacylglycerols and of phospholipids appeared to be increased by hypophysectomy (12). In addition, there was an accumulation of cholesteryl esters and glyceryl-ether diesters and an increase in C 22:5 in both classes. Administration of gonadotropins partially prevented the effect of hypophysectomy.

Prolactin and prolactin plus testosterone caused a reduction in total testicular lipids of control rats (13). Testicular phospholipids, particularly phosphatidyl choline and phosphatidyl ethanolamine, were markedly depleted.

This study was undertaken to determine the effect of hypophysectomy and of replacement therapy with testosterone on the lipid composition and on the metabolism of polyenoic acids in testes of young adult rats at short time periods after hypophysectomy and prior to degeneration of the testes.

MATERIALS AND METHODS

Animals

Sprague-Dawley rats (procured from Harlan Sprague-Dawley, Inc., Indianapolis, Indiana) of 13 weeks of age were used. Rats were divided into two groups. One group remained intact

*To whom correspondence should be addressed.

and served as controls. The other group was hypophysectomized by the transaural route. The hypophysectomized rats were divided into two groups. One group was injected subcutaneously with testosterone (Sigma Chemical Company, St. Louis, Missouri) and the other group was injected only with the carrier, propylene glycol. The exact dosages and the length of time of replacement therapy are detailed in the section on results for a particular set of data.

Some of the rats of each group were injected intratesticularly with a ^{14}C-polyene substrate complexed with albumin. The following radioisotopic compounds were used: 1 μCi per testis of 1-[^{14}C]linoleate (Amersham Corp., Arlington Heights, Illinois, 56 mCi/m mol), 1 μCi per testis of 1-[^{14}C]eicosa-8,11,14-trienoate (New England Nuclear Corp., Boston, Massachusetts, 54.9 mCi/m mol) or 1 μCi per testis of 1-[^{14}C]-arachidonate (Amersham Corp., 57.6 mCi/m mol). The rats were killed at four or 24 hours after [^{14}C]-substrate administration and the testes removed immediately, chilled, weighed and extracted.

Analyses of Lipids

Testes were homogenized in chloroform/methanol (2:1, v/v) for preparation of a total lipid extract. Aliquots of the total lipid extract were used for the determination of total and free cholesterol by the method of Sperry and Webb (14), of total phospholipids by the method of Doizaki and Zieve (15) and of acylglycerols by the method of Soloni (16).

Transesterification of total lipids and of lipid classes was done by the method of Morrison and Smith (17). Analytical gas chromatography of methyl esters was done using a Varian Aerograph model 1520 gas chromatograph equipped with flame ionization detectors and a column packed with 10% SP 2340 on 110/120 mesh Supelcoport. The temperature was programmed from 175 to 225 C at 5 C/min. Known standards were used for qualitative and quantitative calibration. Measurement of peak areas was done using a Hewlett-Packard Model 3390A Reporting Integrator.

Lipid Class Separations

Methods for thin-layer chromatography of total lipids have been reported previously (18). Lipid classes were identified by using known standards spotted on a lane next to the samples. For ^{14}C determination the silica gel containing each lipid class was scraped directly into a counting vial. For gas chromatographic analysis of fatty acids the silica gel was transferred to a teflon-lined, screw-capped tube, and the lipid transesterified according to the method of Morrison and Smith (17).

^{14}C Incorporation

Radioisotopic determination of total lipids and of fatty acids was done using a Packard Tricarb Model 3000 spectrometer. Separation of fatty acid methyl esters for ^{14}C determination was accomplished as reported previously (19) using a Varian model 1800 gas chromatograph equipped with a thermal conductivity cell, the exit port of which was connected to a heated Packard Instrument Company fraction collector. The methyl esters were trapped in glass wool packed into glass cartridges. The glass wool and cartridges were transferred to a counting vial for ^{14}C determination. Eighty to ninety percent of the radioactivity injected into the column was usually recovered in the fatty acids collected.

Statistical Analysis

Data were analyzed for statistical significance by the two-sample student's t-test (20).

RESULTS

In hypophysectomized rats compared to control rats the weights of the body, adrenal glands, prostate and seminal vesicles were decreased significantly at eight days after hypophysectomy (77, 38, 29 and 44%, respectively, of the values for control rats). The weight of the testes was decreased less than 20% (3.60 ± 0.06 vs. 2.90 ± 0.05 g). Treatment with testosterone at 0.5 mg per day for eight days starting at day 1 of hypophysectomy maintained the weights of the prostate and seminal vesicles (80% of the values for control rats for both organs). However, treatment with testosterone at 17 μg/100 gm body weight per day or 1 mg per day for four days starting at day 5 after hypophysectomy did not influence the weights of the accessory sex organs. The weight of the testes was not affected.

The concentration of testicular phospholipids was not significantly different among control, hypophysectomized and hypophysectomized rats treated with 0.5 mg testosterone. However, the concentrations of triacylglycerols, total and unesterified cholesterol were significantly increased in hypophysectomized rats compared to control rats at the eighth day posthypophysectomy (Table 1). Treatment with testosterone at 0.5 mg per day for eight days starting on the day of hypophysectomy was effective in restoring the concentrations of these lipids to normal levels

TABLE 1

Concentration of Lipid Classes in Testes of Control, Hypophysectomized and Hypophysectomized Rats Treated with Testosterone*

	Phospholipids	Triacylglycerols	Total cholesterol	Unesterified cholesterol
Control (6)	13.4 ± 0.20	2.00 ± 0.04	1.73 ± 0.02	1.45 ± 0.02
Hypophysectomized (7)	13.6 ± 0.23	2.53 ± 0.14[a]	1.98 ± 0.05[a]	1.55 ± 0.03[b]
Hypophysectomized-testosterone treated† (7)	13.2 ± 0.21	1.90 ± 0.04[c]	1.70 ± 0.03[d]	1.34 ± 0.02[d]

*Results are expressed as mg per g wet weight of testis; mean ± S.E.; figures in parentheses are the number of samples.

†Hypophysectomized rats injected subcutaneously with 0.5 mg testosterone daily for eight days starting on the first day of hypophysectomy.

[a]Different from control rats $P<0.005$.

[b]Different from control rats $P<0.01$.

[c]Different from hypophysectomized rats $P<0.001$.

[d]Different from hypophysectomized rats $P<0.0005$.

TABLE 2

Fatty Acid Composition of Phospholipids and of Triacylglycerols in Testes of Control and Hypophysectomized Rats*

	Phospholipids		Triacylglycerols	
Fatty acid	Control (6)	Hypophysectomized (7)	Control (6)	Hypophysectomized (7)
16:0	37.9 ± 0.39	38.5 ± 0.45	33.0 ± 0.47	32.9 ± 0.62
18:0	7.8 ± 0.43	6.2 ± 0.04[a]	2.5 ± 0.07	3.1 ± 0.11[a]
18:1	12.2 ± 0.21	12.7 ± 0.25	12.3 ± 0.59	11.4 ± 0.43
18:2	4.5 ± 0.10	3.5 ± 0.05[b]	2.8 ± 0.23	2.9 ± 0.39
20:3	0.75 ± 0.03	0.86 ± 0.03[c]	1.3 ± 0.05	1.8 ± 0.05[b]
20:4	16.7 ± 0.34	16.8 ± 0.20	3.9 ± 0.09	3.6 ± 0.11
22:4	0.95 ± 0.02	0.72 ± 0.02[b]	4.9 ± 0.04	3.8 ± 0.11[b]
22:5	15.8 ± 0.23	17.4 ± 0.28[a]	29.6 ± 0.69	31.7 ± 0.62[d]
22:6	1.1 ± 0.09	1.1 ± 0.04	3.1 ± 0.09	2.8 ± 0.11
24:4	0.52 ± 0.03	0.37 ± 0.01	4.5 ± 0.08	3.9 ± 0.09[b]

*Results are expressed as weight percent; mean ± S.E.; figures in parentheses are the number of samples; not all the fatty acids are listed; therefore, these percentages do not total 100%.

[a]Different from control rats $P<0.005$.

[b]Different from control rats $P<0.0005$.

[c]Different from control rats $P<0.01$.

[d]Different from control rats $P<0.025$.

(Table 1). In contrast, treatment with testosterone at 17 μg per 100 g body weight per day or at 1 mg per day for four days starting at day 5 posthypophysectomy was not effective in restoring the concentrations of these lipids to normal values (data not shown). The concentrations of total and unesterified cholesterol, but not of phospholipids and triacylglycerols, were significantly increased in hypophysectomized compared to control rats 14 days posthypophysectomy. None of these changes were observed at earlier time periods (four and six days) after hypophysectomy (data not shown).

The fatty acid composition of total phospholipids and of triacylglycerols of hypophysectomized rats was compared with that of the control rats at eight days posthypophysectomy. The results are given in Table 2. Testicular phospholipids of hypophysectomized rats had more C 20:3 and C 22:5 and less C 18:0, C 18:2 and C 22:4 than did those of control rats. The fatty acid composition of total lipids was similar to that of the phospholipid fraction (data not shown). Triacylglycerols of testes of hypophysectomized rats had more C 18:0, C 20:3 and C 22:5 and less C 22:4 than did those of control rats. Treatment of hypophysectomized rats with testosterone at all dosages described above had no influence on the fatty acid composition of either phospholipids or

triacylglycerols.

The incorporation of ^{14}C-linoleate into lipid classes of control and hypophysectomized rats on the eighth day posthypophysectomy was determined at four and 24 hours after intratesticular injection. The results are given in Table 3. Most of the radioactivity was recovered in the phospholipid fraction. However, there was a smaller proportion of ^{14}C in testicular phospholipids of hypophysectomized rats than in those of control rats. This was paralleled by a greater proportion of ^{14}C in triacylglycerols of testes of hypophysectomized compared to control rats.

The incorporation of ^{14}C from ^{14}C-linoleate into fatty acids of phospholipids and of triacylglycerols of testes from these rats was determined. Differences between control and hypophysectomized rats were observed at four and 24 hours following injection of the labeled substrate. Because the values for incorporation of isotope into the various fatty acids were greater at 24 than at four hours, the results for the former studies are given in Table 4. Testicular phospholipids of hypophysectomized rats had more ^{14}C in C 16:0, C 20:2 and C 20:3 and less ^{14}C in C 20:4 and C 22:4 than did those of control rats. Similar differences were observed in the fatty acids of the triacylglycerol fraction, but, in addition, there was less ^{14}C in C 18:2 and in C 22:5 in triacylglycerols of hypophysectomized rats than in control rats. Administration of testosterone had no influence on the results of the metabolism of [^{14}C]linoleate

TABLE 3

Incorporation of ^{14}C From 1-[^{14}C] Linoleate into Lipid Fractions of Testes of Control and Hypophysectomized Rats*

	Time of incorporation(h)			
	4		24	
Lipid class	Control (5)	Hypophysectomized (7)	Control (4)	Hypophysectomized (6)
Phospholipids	89.0 ± 0.44	79.5 ± 0.67	86.7 ± 0.23	74.9 ± 1.5
Unesterified cholesterol	2.6 ± 0.20	2.4 ± 0.19	2.9 ± 0.09	3.4 ± 0.23
Unesterified fatty acids	0.86 ± 0.08	5.2 ± 0.75	0.69 ± 0.10	1.1 ± 0.16
Triacylglycerols	6.4 ± 0.23	11.0 ± 0.45	8.2 ± 0.4	15.4 ± 1.0
Cholesteryl esters	0.61 ± 0.05	1.4 ± 0.11	0.76 ± 0.01	2.5 ± 0.24

*Results are expressed as % of total ^{14}C recovered from thin-layer plate; mean ± S.E.; figures in parentheses are the number of samples. [^{14}C]linoleate was injected intratesticularly on the eighth day posthypophysectomy and the rats killed 24 hours after the ^{14}C administration.

TABLE 4

Incorporation of ^{14}C From 1-[^{14}C] Linoleate into Fatty Acids of Phospholipids and of Triacylglycerols of Testes of Control and Hypophysectomized Rats*

	Phospholipids		Triacylglycerols	
Fatty acid	Control (4)	Hypophysectomized (6)	Control (4)	Hypophysectomized (6)
16:0	3.8 ± 0.21	10.6 ± 0.63[a]	7.6 ± 0.45	16.2 ± 1.1[a]
18:2	62.6 ± 1.2	59.3 ± 1.1	45.5 ± 1.7	30.2 ± 2.4[b]
20:2	2.2 ± 0.11	3.8 ± 0.22[a]	4.9 ± 0.21	8.2 ± 0.23[a]
20:3	5.7 ± 0.05	7.8 ± 0.27[a]	11.1 ± 0.39	20.7 ± 0.53[a]
20:4	17.6 ± 1.0	9.9 ± 0.30[a]	7.6 ± 0.60	3.8 ± 0.48[b]
22:3	0.88 ± 0.06	0.85 ± 0.07	0.85 ± 0.13	1.9 ± 0.12[a]
22:4	1.5 ± 0.12	1.1 ± 0.04[b]	6.7 ± 0.27	2.0 ± 0.16[a]
22:5	1.0 ± 0.03	1.2 ± 0.06	3.8 ± 0.19	2.0 ± 0.16[a]

*Results are expressed as % of ^{14}C recovered in fatty acids of each particular lipid fraction; mean ± S.E.; figures in parentheses are the number of samples; not all the fatty acids are listed; therefore, these percentages do not total 100%. [^{14}C]linoleate was injected intratesticularly on the eighth day posthypophysectomy and the rats killed 24 hours later.

[a]Different from control rats $P<0.0005$.

[b]Different from control rats $P<0.005$.

either at four or 24 hours after injection.

^{14}C incorporation from intratesticularly injected 1-[^{14}C]eicosa-8,11,14-trienoate (C 20:3) into lipids of testes of control and hypophysectomized rats was determined on the eighth day posthypophysectomy. The rats were killed four hours after the ^{14}C injection. In Table 5 are given results of determinations of the ^{14}C in different lipid classes. There was less ^{14}C in testicular phospholipids of hypophysectomized rats than in those of control rats, and this was paralleled by a greater ^{14}C content in testicular triacylglycerols of hypophysectomized than in those of control rats.

Results of studies of ^{14}C incorporation from this substrate into the fatty acids of phospholipids and triacylglycerols in these rats are shown in Table 6. There was more ^{14}C in C 16:0 and C 20:3 and less ^{14}C in C 20:4 and C 22:4 in testicular phospholipids of hypophysectomized rats than in those of control rats. Similar results were obtained in the triacylglycerol fraction, but there was also decreased ^{14}C in C 22:5 in the hypophysectomized rats.

Treatment with 0.5 mg testosterone per day for eight days beginning on the first day of hypophysectomy did not affect any of the results of the metabolism of [^{14}C]eicosatrienoate in hypophysectomized rats.

Similar studies were done using 1-[^{14}C]-arachidonate as the injected substrate. Results of studies of ^{14}C incorporation into lipid classes

TABLE 5

Incorporation of ^{14}C From 1-[^{14}C]Eicosa-8,11,14-Trienoate and From 1-[^{14}C]-Arachidonate into Lipid Fractions of Testes of Control and Hypophysectomized Rats*

	^{14}C-compound injected			
	1-[^{14}C]eicosatrienoate		1-[^{14}C]arachidonate	
	Control (6)	Hypophysectomized (7)	Control (6)	Hypophysectomized (6)
Phospholipids	86.0 ± 0.78	78.7 ± 1.1[a]	89.3 ± 0.35	90.2 ± 0.14
Unesterified cholesterol	2.5 ± 0.33	1.7 ± 0.09	3.1 ± 0.26	2.5 ± 0.14
Unesterified fatty acids	4.2 ± 0.47	4.8 ± 1.0	1.3 ± 0.13	1.2 ± 0.28
Triacylglycerols	6.2 ± 0.76	12.3 ± 0.32[a]	5.2 ± 0.19	4.9 ± 0.2
Cholesteryl esters	0.66 ± 0.09	1.8 ± 0.19	0.73 ± 0.06	0.65 ± 0.09

*Results are expressed as % of total ^{14}C recovered from thin-layer plate; mean ± S.E.; figures in parentheses are the number of samples. [^{14}C]eicosatrienoate or [^{14}C]arachidonate was injected intratesticularly on the eighth day posthypophysectomy and the rats killed four hours after the ^{14}C administration.

[a]Different from control rats $P<0.0005$.

TABLE 6

Incorporation of ^{14}C From 1-[^{14}C]Eicosa-8,11,14-Trienoate into Fatty Acids of Phospholipids and of Triacylglycerols of Testes of Control and Hypophysectomized Rats*

	Phospholipids		Triacylglycerols	
Fatty acid	Control (6)	Hypophysectomized (7)	Control (6)	Hypophysectomized (7)
16:0	1.8 ± 0.13	2.3 ± 0.21[a]	8.0 ± 0.61	9.6 ± 0.75
20:3	37.4 ± 1.0	76.5 ± 1.3[b]	52.1 ± 2.8	77.2 ± 1.8[b]
20:4	53.7 ± 0.97	15.1 ± 0.96[b]	15.7 ± 0.92	3.3 ± 0.10[b]
22:3	1.2 ± 0.06	1.3 ± 0.25	1.8 ± 0.14	2.5 ± 0.20[c]
22:4	2.3 ± 0.13	0.90 ± 0.11[b]	9.3 ± 0.48	1.7 ± 0.14[b]
22:5	0.64 ± 0.06	0.54 ± 0.12	2.9 ± 0.45	0.50 ± 0.07[b]

*Results are expressed as % of ^{14}C recovered in fatty acids of each particular lipid fraction; mean ± S.E; figures in parentheses are the number of samples; not all the fatty acids are listed; therefore, these percentages do not total 100%. The ^{14}C-substrate was injected intratesticularly on the eighth day posthypophysectomy and the rats killed four hours later.

[a]Different from control rats $P<0.05$.

[b]Different from control rats $P<0.0005$.

[c]Different from control rats $P<0.025$.

TABLE 7

Incorporation of ^{14}C From 1-[^{14}C]Arachidonate into Fatty Acids of Phospholipids and of Triacylglycerols of Testes of Control and Hypophysectomized Rats*

Fatty acid	Phospholipids		Triacylglycerols	
	Control (6)	Hypophysectomized (6)	Control (6)	Hypophysectomized (7)
16:0	1.0 ± 0.05	1.0 ± 0.06	6.7 ± 0.28	8.2 ± 0.38[b]
20:4	88.2 ± 0.51	87.6 ± 0.70	44.3 ± 0.64	46.6 ± 1.5
22:3	1.4 ± 0.11	1.4 ± 0.12	1.0 ± 0.16	0.86 ± 0.15
22:4	4.3 ± 0.08	3.6 ± 0.08[a]	21.1 ± 0.56	18.5 ± 0.60[a]
22:5	1.2 ± 0.12	1.4 ± 0.05	5.8 ± 0.23	5.1 ± 0.23

*Results are expressed as % of ^{14}C recovered in fatty acids of each particular lipid fraction; mean ± S.E; figures in parentheses are the number of samples; not all the fatty acids are listed; therefore, these percentages do not total 100%. [^{14}C]arachidonate was injected intratesticularly on the eighth day posthypophysectomy and the rats killed four hours later.

[a]Different from control rats $P<0.0005$.

[d]Different from control rats $P<0.01$.

are given in Table 5. There were no significant differences between control and hypophysectomized rats in the incorporation of ^{14}C into the different lipid fractions. Results of studies of ^{14}C incorporation into fatty acids of phospholipids and of triacylglycerols are summarized in Table 7. There was a slight but statistically significant decrease in the amount of ^{14}C in C 22:4 of testicular phospholipids and triacylglycerols of hypophysectomized compared to control rats.

DISCUSSION

The effectiveness of the hypophysectomy was confirmed by the decrease in weight of the adrenal glands, prostate and seminal vesicles. Treatment with testosterone at 0.5 mg per day for eight days starting at day one posthypophysectomy prevented the changes in the accessory sex organs. This treatment also resulted in higher serum testosterone levels than those of hypophysectomized rats not treated with testosterone (16.7 ± 2.5 vs 1.5 ± 0.53 ng/ml).

Significant changes in testicular triacylglycerols due to hypophysectomy were observed at 8 days posthypophysectomy but not at 4, 6 or at 14 days. At the same time there was an increased concentration in cholesterol in the testes of hypophysectomized rats compared to the control rats. This probably was due to decreased steroid synthesis in the absence of luteinizing hormone. This also led to an increase in cholesteryl esters, the storage form of cholesterol. At least one of the schedules of testosterone therapy used was effective in the maintenance of normal levels of these lipids.

The concentration of testicular phospholipids was not affected by hypophysectomy during the time periods studied. Our results are thus similar to those of Gambal and Ackerman (9), whose studies covered a period of 20 days after hypophysectomy.

Hypophysectomy also resulted in significant changes in the fatty acid composition not only of triacylglycerols but also of the phospholipids. Of major interest is the effect on polyenoic acids of the linoleate series. The concentration of the 22-carbon pentaene (C 22:5) of this series increases three-fold in rat testis during sexual maturation (21,2), and the pentaene may be associated with normal development of spermatids (22,23). The concentration of C 22:5 was greater in testes of hypophysectomized rats compared to those of the control group. Although the concentration of C 20:3 of this series was also greater in testes of hypophysectomized compared to control rats, the concentrations of C 22:4, the immediate precursor of C 22:5, and of linoleate, the first member of this series and a dietary essential, were lower than those in testes of control rats. Administration of testosterone in the three methods described in a preceding section did not alter these changes.

In order to determine the reason for the changes in fatty acid composition brought about by hypophysectomy, a number of ^{14}C-labeled substrates in this sequence of reactions (C 18:2 to C 22:5) was used. When [^{14}C]-linoleate was injected intratesticularly, there was an accumulation of ^{14}C in C 20:3 and a decreased amount of ^{14}C in C 20:4 in testes of hypophysectomized compared to control rats. In the triacylglycerol fraction there also was a smaller amount of ^{14}C in the linoleate fraction of the hypophysectomized compared to the control rats. These data suggested that there was no impairment in the conversion of C 18:2 to higher polyenes but that there might be an

impairment in the conversion of C 20:3 to other polyenes (particularly to arachidonic acid).

The conversion of C 20:3 to C 20:4 was investigated in the two groups of rats more directly by use of intratesticularly-injected [^{14}C] 20:3. In both triacylglycerols and phospholipids of testes of hypophysectomized rats the amount of ^{14}C in C 20:3 was much greater than that found in the two lipid fractions of testes of control rats. These results confirmed those suggested by the studies using [^{14}C] linoleate and pointed to an inhibition of the Δ5 desaturase in the hypophysectomized rats.

Following the intratesticular injection of [^{14}C]linoleate and [^{14}C] 20:3 less ^{14}C was incorporated into C 22:4 in testes of hypophysectomized rats than in those of control rats. This result could be due either to a decrease in the availability of the precursor, [^{14}C]arachidonate, in the testes of the hypophysectomized rats, or to an inhibition of the elongation of C 20:4 to C 22:4. The substrate [^{14}C]arachidonate was used to distinguish between these two possibilities. There was only a slight decrease in ^{14}C incorporation into C 22:4 of testicular lipids of the hypophysectomized rats, and this was not statistically significant when expressed on the basis of testicular weight. Therefore, the data indicate that the elongation of C 20:4 to C 22:4 was not affected by the hypophysectomy.

Following intratesticular injection of the three [^{14}C] substrates the amount of ^{14}C found in C 22:5 in phospholipids was similar in the testes of the two groups. However, after injection of [^{14}C]linoleate and [^{14}C]arachidonate but not after [^{14}C]eicosatrienoate there was a smaller amount of ^{14}C in C 22:5 of testicular triacylglycerols of hypophysectomized compared to control animals. These data do not permit any conclusions concerning the effect of hypophysectomy on the Δ4 desaturase (C 22:4 to C 22:5). The testicular metabolism of C 22:5 (as well as of C 22:4) is complicated because, in addition to opposing reactions of biosynthesis and of degradation by beta oxidation to acetyl CoA, there is an active process of retroconversion in which 22:5 is converted to 22:4 and subsequently to 20:4 (24).

When [^{14}C]linoleate was used for intratesticular injection, there was an accumulation of ^{14}C in C 20:2 as well as C 20:3 in testicular lipids of hypophysectomized rats. Rat liver (25) and brain (26) do not have Δ8 desaturase activity and desaturate eicosa-11,14-dienoic acid to eicosa-5,11,14-trienoic acid, a dead-end product. However, rat testis can desaturate this dienoic acid to the 8,11,14 isomer as well as the 5,11,14 isomer (27). It is possible, therefore, that this pathway may be stimulated in the testes of hypophysectomized rats or that the accumulation of C 20:3 inhibited the conversion of C 20:2 to the trienoic acid.

Treatment with testosterone at any of the dosages described previously had no effect on the incorporation of ^{14}C in testes of the hypophysectomized rats. It is possible that treatment using other dosages or schedules of administration or that the combined use of testosterone and some other hormone may have an influence on these reactions involving desaturation of substrates. In this regard it is relevant to note that inhibition of liver, kidney and aorta Δ5 desaturase has been reported in rats made diabetic by streptozotocin (28).

In contrast to the results obtained on ^{14}C incorporation into the polyenoic acids in hypophysectomized rats treated with testosterone were those in which the elevated levels of triacylglycerol and cholesterol observed in testes of hypophysectomized rats were prevented by administration of testosterone. It appears, therefore, that testosterone alone may be effective in controlling some aspects of lipid metabolism.

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REFERENCES

1. Coniglio, J.G. (1977) in The Testis, Vol. IV (Johnson, A.D. and Gomes, W.R., eds.), pp. 425-449, Academic Press, New York.
2. Davis, J.T.; Bridges, R.B.; and Coniglio, J.G. (1966) Biochem. J. 98, 342-346.
3. Beckman, J.K. and Coniglio, J.G. (1979) Lipids 14, 262-267.
4. Davis, J.T. and Coniglio, J.G. (1966) J. Biol. Chem. 241, 610-612.
5. Burr, G.O. and Burr, M.M. (1929) J. Biol. Chem. 82, 345-367.
6. Burr, G.O. and Burr, M.M. (1930) J. Biol. Chem. 86, 587-621.
7. Smith, P.E. (1927) J. Clin. Med. Assoc. 88, 158-161.
8. Smith, P.E. (1930) Am. J. Anat. 45, 205-273.
9. Gambal, D. and Ackerman, R.J. (1967) Endocrinology 80, 231-239.
10. Nakamura, M.; Jensen, B.; and Privett, O.S. (1968) Endocrinology 82, 137-142.
11. Goswami, A. and Williams, W.L. (1967) Biochem. J. 105, 537-543.
12. Nakamura, M. and Privett, O.S. (1973) Lipids 8, 224-231.

13. Carney, J.A. and Walker, B.L. (1971) Nutr. Reports Int. 4, 103-108.
14. Sperry, W.M. and Webb, M. (1950) J. Biol. Chem. 187, 97-106.
15. Doizaki, W.M. and Zieve, L. (1963) Proc. Soc. Exp. Biol. Med. 113, 91-94.
16. Soloni, F.G. (1971) Clin. Chem. 17, 529-534.
17. Morrison, W.R. and Smith, L.M. (1964) J. Lipid Res. 5, 600-608.
18. Beckman, J.K.; Gray, M.E.; and Coniglio, J.G. (1978) Biochim. Biophys. Acta 530, 367-374.
19. Beckman, J.K. and Coniglio, J.G. (1980) Lipids 15, 389-394.
20. Larsen, R.J. (1975) Statistical Analysis for the Allied Health Sciences, pp. 177-194, Charles E. Merrill, Columbus.
21. Kirschman, J.C. and Coniglio, J.G. (1961) Arch. Biochem. Biophys. 93, 297-301.
22. Coniglio, J.G.; Whorton, A.R.; and Beckman, J.K. (1977) in Function and Biosynthesis of Lipids (Bazan, N.G., Brenner, R.R. and Giusto, M.M., eds.), pp. 575-589, Plenum Press, New York.
23. Coniglio, J.G.; Grogan, W.M.; Morris, D.G., and Fitzhugh, M.L. (1975) Lipids 10, 109-112.
24. Bridges, R.B. and Coniglio, J.G. (1970) Biochim. Biophys. Acta 218, 29-35.
25. Ullman, D. and Sprecher, H. (1971) Biochim. Biophys. Acta 248, 186-197.
26. Dhopeshwarkar, G.A. and Subramanian, C. (1976) J. Neurochem. 26, 1175-1179.
27. Albert, D.H. and Coniglio, J.G. (1977) Biochim. Biophys. Acta 489, 390-396.
28. Holman, R.T.; Johnson, S.B.; Gerrard, J.M.; Mauer, S.M.; Kupcho-Sandberg, S.; and Brown, D.M. (1983) Proc. Nat'l. Acad. Sci., U.S.A. 80, 2375-2379.

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Alteration and Recovery of Bleeding Times, Platelet Aggregation and Fatty Acid Composition of Individual Phospholipids in Platelets of Human Subjects Receiving a Supplement of Cod-Liver Oil

AHMED A. AHMED and **BRUCE J. HOLUB***, *Department of Nutrition, College of Biological Science, University of Guelph, Guelph, Ontario, Canada N1G 2W1*

ABSTRACT

The effect of supplementation with cod-liver oil containing eicosapentaenoic acid (EPA), 20:5ω3, on bleeding times, thrombin-induced platelet aggregation, platelet protein, platelet cholesterol, and the level and fatty acid composition of individual phospholipids in the platelets of human subjects was determined. Measurement of these parameters was conducted before the subjects received the supplement (day 0), after they received the supplement for 14 days (day 14), and 14 days after the supplement was terminated (day 28) so as to monitor recovery. The mean bleeding times exhibited a marked increase (by 81%) with supplementation and returned to near basal (day 0) values within 14 days after the supplement was terminated. Cod-liver oil supplementation significantly reduced thrombin-induced platelet aggregation with a partial recovery being exhibited by day 28. The content of phospholipid, cholesterol and protein ($\mu g/10^9$ platelets) was not significantly different ($P>0.05$) when isolated from the subjects at day 0, 14 and 28, as neither were the composition of individual phospholipids [phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI) and sphingomyelin (SPH)] given as % of total phospholipid. However, the fatty acid compositions of all platelet phospholipids were altered significantly by the fish oil supplement. In PC, EPA rose from 0.3 to 2.9% of total fatty acids and docosahexaenoate from 0.7 to 1.8% concomitant with a drop in arachidonate (from 14.1 to 9.6%) and linoleate (from 10.2 to 7.9%); these levels approached basal levels 14 days after supplementation was terminated. The highest percentage of EPA with supplementation was found in PE (4.3%), while the arachidonate fell from 38.8 to 30.5%, with low percentages of EPA occurring in PS (0.7%) and PI (0.5%). The level of 24:1 in SPH increased significantly (from 17.8 to 24.8) with supplementation and reverted to basal values by day 28. These results suggest a close relationship of the observed fatty acid changes in individual platelet phospholipids to the altered hematological parameters and platelet-vessel wall interactions produced by cod-liver oil supplementation.

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INTRODUCTION

The consumption of a diet enriched in fish or fish oil containing EPA, 20:5ω3, recently has been shown to diminish platelet aggregation and prolong bleeding times in human subjects (1-4). Dyerberg and Bang (2) have suggested that the ingestion of EPA in significant quantities by the Greenland Eskimo may protect them from arterial thrombosis and atherosclerotic cardiovascular disease. A dietary supplement of marine oil was found to reduce the incidence of experimental cerebral infarction in cats and myocardial infarction in dogs (5,6). Recently, the consumption of a daily supplement of cod-liver oil in patients with ischemic heart disease was found to produce changes in platelet kinetics which would limit the progression of atheroma and reduce platelet/vessel-wall interactions (7).

The mechanisms by which dietary EPA decreases platelet aggregation have not been clearly established, although a few proposals have been made (1,4). Included among these are an increased EPA/arachidonate ratio in the phospholipid of platelet membranes, a reduced release of arachidonate from the phospholipids of stimulated platelets, and/or a reduced formation of thromboxane A_2 from the liberated arachidonate via competitive inhibition of EPA at the level of the cyclo-oxygenase. The consumption by human subjects of diets containing fish or supplemented with fish oil for 1 to 11 weeks produces an elevation in the percentage of EPA in the fatty acids of platelet phospholipid (3,4,8-11). The effect of such dietary alterations on the level and relative distribution of individual phospholipids in the human platelet was not studied in these former studies nor was the effect of fish oil supplementation on the fatty acid composition of human platelet PI apart from total or other phospholipids such as PS. PI has been impli-

*To whom correspondence should be addressed.

cated in platelet aggregation and the release of arachidonic acid for conversion to prostaglandins including thromboxane A_2 (12-16). Other work has emphasized the potential importance of PC and PE as sources of free arachidonate in stimulated platelets (15,17-19).

The purpose of the present work was to study the effect of cod-liver oil supplementation on bleeding times, platelet aggregation (thrombin-induced), and the level and fatty acid composition of individual phospholipids in the platelets of human subjects and the recovery of these same parameters after terminating supplementation.

MATERIALS AND METHODS

Subjects and Experimental Design

The subjects were seven healthy volunteers having a mean age of 32 yr. They were taking no medication and were asked to maintain their normal dietary habits over the period of study. Informed consent was obtained from the subjects, and the study received certification of ethical acceptability for research involving human subjects from the review committee of the University of Guelph. During the period of supplementation, the subjects consumed 10 ml of cod-liver oil (Life Brand, Toronto, Ontario, Canada) containing EPA (6%) and docosahexaenoic acid (6%) plus other fatty acids three times daily with meals (30 ml/day) for 2 wk.

Blood samples were taken for platelet isolation, aggregation studies and biochemical analyses on three different occasions from each of the subjects—at day 0 (just before receiving the cod-liver oil supplement), after receiving the supplement continually for 14 days (day 14), and after having terminated supplementation for a period of 14 days (day 28)—28 days following initiation of the trial. The latter sampling allowed a comparison of the various platelet parameters (recovery values) with basal values obtained at day 0. At each time when blood samples were taken, bleeding times were performed by the modified Duke procedure (20) following puncture of the ear lobe.

Platelet Isolation and Aggregation

Approximately 27 ml of blood was drawn from the antecubital veins into siliconized vacutainer tubes containg 1/10 volume of 3.8% sodium citrate as anti-coagulant. The blood was spun at 111xg for 15 min at room temperature to obtain platelet-rich plasma (PRP). The platelets were then pelleted from PRP according to a procedure similar to that of McKean et al. (18) in which PRP containg 6 mM EDTA is centrifuged at 1100xg for 15 min at 4 C to remove the platelet-poor plasma (PPP). The platelets were resuspended in one volume of Tris-saline buffer (20 mM Tris-HCl, pH 7.4, and 150 mM NaCl containing 2 mM EDTA) and sedimented by centrifugation at 1000xg for 10 min at 4 C. The platelets were finally resuspended in 1/3 volume of Tris-saline buffer containing 5 mM glucose.

Platelet Counting and Protein Analyses

Cell counts were performed on the final platelet suspension by phase contrast microscopy using a standard microscope with in-base halogen illuminator 6V10W (Carl Zeiss, Oberkochen, West Germany). Platelet protein was determined by the method of Lowry et al. (21) on duplicate 10 μl aliquots of the final platelet suspensions, using bovine serum albumin as the standard.

Platelet Aggregation

Platelet aggregation was performed by the method of Born (22) at 37 C in an aggregometer cuvette by stirring 0.5 ml of the final platelet suspensions at 900 rpm. The extent of aggregation was measured as the maximal transmission of light (in per cent) using a dual-channel Payton aggregometer (Payton Associates Ltd., Scarborough, Ontario, Canada). Platelet samples were tested in each subject at day 0, day 14, and day 28 (see above). Thrombin (Sigma Chemical Co., St. Louis, Missouri) was added to provide a concentration of 0.8 units/ml.

Platelet Lipid Analyses

Platelet lipids were extracted according to the method of Folch et al. (23) using 10 ml of chloroform:methanol (2:1, v/v) for each 2 ml of platelet suspension. Platelet cholesterol was determined on aliquots of the lipid extracts as described (24). The remainder of the lipid extracts were applied to glass plates coated with a 0.5 mm layer of Silica gel H (Analabs, North Haven, Colorado) following activation overnight at 115 C. The plates were developed in a system modified from that of Skipski et al. (25) using chloroform:methanol:acetic acid:water (50:30:5:3, v/v/v/v) to separate individual phospholipids. The chromatograms were visualized under ultraviolet light after being sprayed with a solution of dichlorofluorescein in water: methanol (50:50, v/v) and exposed to ammonia vapor.

The amount and fatty acid compositions of the individual phospholipids, PC, PE, PS, PI, and SPH, were determined by gas-liquid chromatography after transmethylation in the presence of the gel scrapings and known amounts of

monopentadecanoin (Nu Chek Prep, Elysian, Minnesota), as an internal quantitative standard (26), for 14 hr at 80 C using 6% (by vol) H_2SO_4 in methanol. Corresponding blank regions of the plate also were methylated routinely in the presence of internal standard and analyzed as controls. Gas-liquid chromatographic analyses of fatty acid methyl esters were performed as described by Chapkin et al. (26) on glass columns packed with 10% Silar-10C (Applied Science Laboratories Inc., State College, Pennsylvania) using a Hewlett-Packard 5840A gas chromatograph equipped with flame ionization detectors. Identification of the peaks was established by comparison of retention data with known standards (Nu Chek Prep, Elysian, Minnesota) and by gas-liquid analysis following separation of the fatty acid methyl esters by argentation thin-layer chromatography.

Statistical Analyses

All data obtained on the subjects just before receiving the cod-liver oil supplement (day 0), after receiving the supplement for 14 days (day 14), and after having terminated supplementation for 14 days (day 28) were analyzed by Duncan's multiple range test (27). The level of significance was chosen at $P < 0.05$.

RESULTS

The consumption of the supplement of cod-liver oil containing EPA for 14 days was found to produce a marked increase (by 81%) in the mean bleeding times to a level which was significantly greater ($P < 0.01$) than basal (pre-supplement) values (Figure 1). Within 14 days of terminating the supplement, the bleeding times decreased to levels which were not significantly different ($P > 0.05$) from basal values, although the mean value was 20% above the basal.

Summarizing the platelet aggregation data from all subjects (Figure 2) reveals that the overall aggregation response to thrombin was significantly suppressed ($P < 0.05$), by 53 to 67%, due to the consumption of the fish oil supplement for in vitro aggregation measurements taken 1, 2, and 3 min following thrombin addition. These values approached pre-supplement levels after terminating supplementation for 14 days but did not fully recover to basal values within this interval.

The cellular content of phospholipid, cholesterol and protein ($\mu g/10^9$ platelets) was found not to be significantly different ($P > 0.05$) when isolated from the subjects before receiving, after receiving, and after terminating

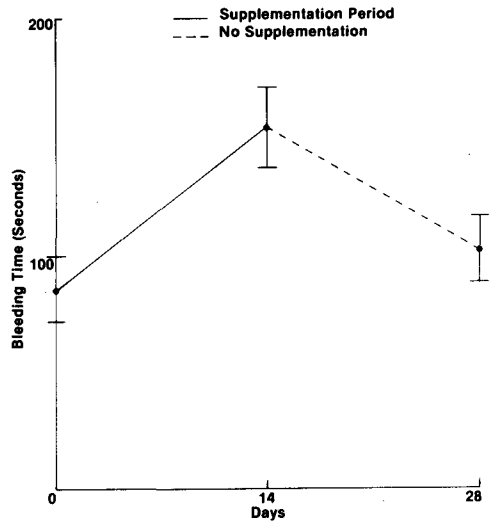

FIG. 1. Effect of cod-liver oil supplementation on bleeding times. Bleeding times were measured before receiving supplement (day 0), after receiving the supplement for 14 days (day 14), and 14 days after terminating the supplement (day 28). All data represent mean values ± SE.

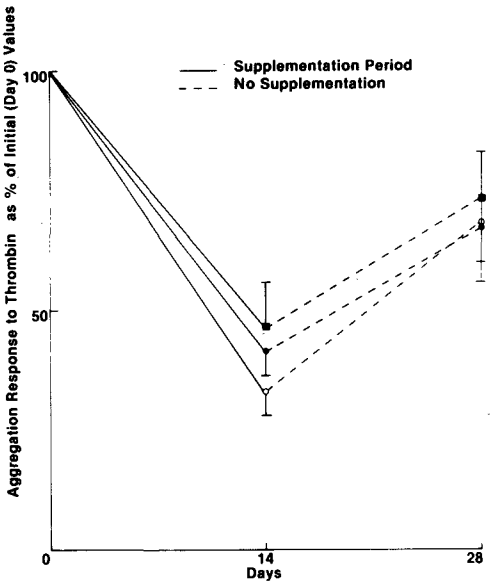

FIG. 2. Effect of cod-liver oil supplementation on aggregation response of platelet suspensions to thrombin. See also legend to FIG. 1.

the supplement of cod-liver oil. The compositional data were in excellent agreement with reported levels of phospholipid, cholesterol and protein in human platelets (28). The composition of individual phospholipids (PC, PE, PS, PI, and SPH) given as % of total phospholipid

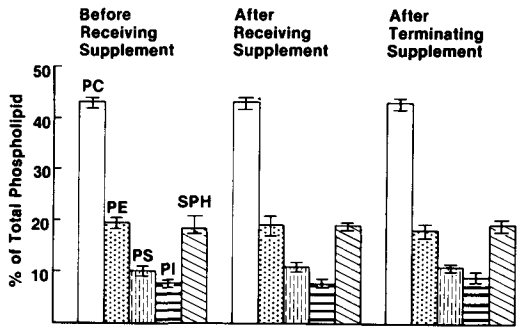

FIG. 3. Effect of cod-liver oil supplementation on phospholipid composition of human platelets. See also legend to FIG. 1. Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine (diacyl plus alkenyl acyl); PS, phosphatidylserine; PI, phosphatidylinositol; SPH, sphingomyelin.

also was not significantly different ($P > 0.05$) at the three different sampling times (Figure 3). The pre-supplement values for the relative abundance of individual phospholipids in human platelets are in general agreement with published data (29-32).

The fatty acid compositions of all five platelet phospholipids were altered significantly by consumption of the fish oil supplement (Tables 1-5). In the case of PC (Table 1), the percentage of EPA rose significantly ($P < 0.05$) in subjects consuming the supplement for 14 days (from 0.3 to 2.9%) concomitant with a marked drop in the level of arachidonate (from 14.1 to 9.6%) and a moderate drop in linoleate (from 10.2 to 7.9%). Within 14 days after terminating supplementation, the levels of EPA, arachidonate and linoleate had approached basal values. The level of docosahexaenoic acid (22:6ω3) also rose significantly with supplementation ($P < 0.05$) and exhibited a partial recovery, but not a complete return to basal levels, after terminating supplementation for 14 days.

The highest enrichment of EPA was found in the PE (Table 2) where it reached 4.3% of the total fatty acids with supplementation while the arachidonate fell from 38.8 to 30.5%. Both fatty acids returned to levels which were not significantly different ($P > 0.05$) from basal values after terminating supplementation. As in the case of phosphatidylcholine, half of the elevation in the 22:6ω3 level due to supplementation was still present 14 days after ceasing the consumption of cod-liver oil.

In contrast to PC and PE, only low levels of EPA accumulated in the PS (0.7%) and PI (0.5%) of the platelets from subjects consuming the cod-liver oil for 14 days (Tables 3 and 4). In the case of PS (Table 3), there was a reduction in the level of arachidonic acid with supplementation which approached, but not completely, pre-supplement levels after terminating supplementation. A reversible doubling of the docosahexaenoate content of platelet PS occurred with fish oil ingestion. Interestingly, the level of arachidonate in platelet PI was only moderately reduced following the consumption of cod-liver oil (Table 4). The level of 20:1 in all four phos-

TABLE 1

Fatty Acid Composition of Phosphatidylcholine in Platelets of Subjects Before Receiving, After Receiving, and After Terminating Supplement of Cod-Liver Oil[1]

Fatty acid	Before receiving supplement	After receiving supplement	After terminating supplement
		(Weight % of total)	
16:0	28.3 ± 0.4[a]	31.2 ± 1.0[b]	27.7 ± 0.4[a]
16:1	1.8 ± 0.1[a]	1.8 ± 0.1[a]	1.5 ± 0.1[b]
18:0	14.4 ± 0.2[a]	13.2 ± 0.3[b]	14.7 ± 0.2[a]
18:1	24.9 ± 0.3[a]	24.5 ± 0.7[a]	24.8 ± 0.3[a]
18:2	10.2 ± 0.4[a]	7.9 ± 0.2[b]	10.5 ± 0.4[a]
20:1	1.6 ± 0.0[a]	2.4 ± 0.1[b]	1.5 ± 0.0[a]
20:2	1.0 ± 0.0[a]	0.9 ± 0.1[a]	1.2 ± 0.1[b]
20:3	1.7 ± 0.1[a]	1.7 ± 0.1[a]	1.9 ± 0.1[a]
20:4	14.1 ± 0.3[a]	9.6 ± 0.3[b]	13.0 ± 0.2[c]
20:5	0.3 ± 0.0[a]	2.9 ± 0.5[b]	0.6 ± 0.1[a]
22:4	0.6 ± 0.1[a]	0.7 ± 0.3[a]	0.7 ± 0.1[a]
22:5	0.5 ± 0.2[a]	1.1 ± 0.4[a]	0.6 ± 0.1[a]
22:6	0.7 ± 0.1[a]	1.8 ± 0.1[b]	1.3 ± 0.0[c]

[1] All data represent mean values ± SE for seven subjects.

Values across each row having different superscript letters are significantly different from each other ($P<0.05$).

Minor fatty acids contributing <0.5% to the total have been omitted from the table.

TABLE 2

Fatty Acid Composition of Phosphatidylethanolamine in Platelets of Subjects Before Receiving, After Receiving, and After Terminating Supplement of Cod-Liver Oil[1]

Fatty acid	Before receiving supplement	After receiving supplement	After terminating supplement
		(Weight % of total)	
16:0	2.7 ± 0.2[a]	4.2 ± 0.5[a]	4.3 ± 0.3[a]
16:0A[2]	6.2 ± 0.9[a]	5.4 ± 1.0[a]	10.2 ± 0.6[b]
16:1	2.3 ± 0.5[ab]	1.4 ± 0.3[a]	2.8 ± 0.3[b]
18:0	16.0 ± 0.6[a]	16.6 ± 1.1[a]	15.6 ± 0.2[a]
18:0A	8.2 ± 0.8[a]	7.9 ± 0.8[ab]	4.5 ± 1.2[b]
18:1	8.0 ± 0.4[a]	9.8 ± 0.8[b]	8.9 ± 0.2[ab]
18:1A	1.5 ± 0.2[a]	1.3 ± 0.2[a]	2.4 ± 0.2[b]
18:2	2.7 ± 0.2[a]	2.9 ± 0.2[a]	3.2 ± 0.2[a]
20:0	0.8 ± 0.2[a]	0.7 ± 0.1[a]	0.7 ± 0.2[a]
20:1	0.4 ± 0.0[a]	1.2 ± 0.2[b]	0.5 ± 0.1[a]
20:3	0.8 ± 0.1[a]	1.0 ± 0.1[a]	0.8 ± 0.1[a]
20:4	38.8 ± 0.9[a]	30.5 ± 1.6[b]	37.2 ± 0.6[a]
20:5	0.5 ± 0.1[a]	4.3 ± 0.6[b]	1.1 ± 0.1[a]
22:4	5.6 ± 0.3[a]	2.7 ± 0.6[b]	4.0 ± 0.2[c]
22:5	2.9 ± 0.3[a]	3.4 ± 0.3[a]	2.8 ± 0.4[a]
22:6	2.1 ± 0.2[a]	4.1 ± 0.4[b]	3.2 ± 0.2[c]

[1] See legend to Table 1.
[2] Aldehyde derivative.

TABLE 3

Fatty Acid Composition of Phosphatidylserine in Platelets of Subjects Before Receiving, After Receiving, and After Terminating Supplement of Cod-Liver Oil[1]

Fatty acid	Before receiving supplement	After receiving supplement	After terminating supplement
		(Weight % of total)	
16:0	0.2 ± 0.3[a]	1.5 ± 0.2[b]	0.6 ± 0.2[a]
16:1	0.3 ± 0.2[a]	1.3 ± 0.2[b]	0.9 ± 0.2[ab]
18:0	39.0 ± 0.7[a]	41.4 ± 0.6[b]	40.2 ± 0.3[ab]
18:1	24.2 ± 1.2[a]	26.2 ± 0.4[a]	25.2 ± 0.4[a]
18:2	3.9 ± 0.3[a]	3.1 ± 0.1[b]	3.8 ± 0.1[a]
20:1	1.3 ± 0.1[a]	2.1 ± 0.1[b]	1.1 ± 0.1[a]
20:2	0.9 ± 0.2[a]	1.1 ± 0.1[ab]	1.3 ± 0.1[b]
20:3	2.1 ± 0.2[a]	2.9 ± 0.1[b]	2.2 ± 0.1[a]
20:4	25.9 ± 1.1[a]	15.9 ± 0.9[b]	21.3 ± 0.3[c]
20:5	0.1 ± 0.1[a]	0.7 ± 0.1[b]	0.2 ± 0.1[a]
22:4	0.8 ± 0.1[a]	0.7 ± 0.1[a]	0.8 ± 0.1[a]
22:5	0.8 ± 0.1[a]	1.0 ± 0.1[a]	0.7 ± 0.1[a]
22:6	1.1 ± 0.2[a]	2.2 ± 0.2[b]	1.4 ± 0.1[a]

[1] See legend to Table 1.

pholipids (Tables 1-4) increased and then returned to basal levels after receiving and after terminating the supplement, respectively. It is of interest that a fully reversible and statistically significant ($P < 0.05$) increase in the level of 24:1 (from 17.8 to 24.8%) and decrease in the level of 22:0 (from 24.2 to 19.8%) in SPH (Table 5) resulted from the cod-liver oil supplement despite the fact that this phospholipid did not contain any significant amount of EPA or arachidonic acid.

DISCUSSION

The marked increase (by 81%) in mean overall capillary bleeding times as measured by the modified Duke procedure concomitant with the reduction in thrombin-induced platelet aggregation, measured using washed platelet suspensions due to the ingestion of a cod-liver oil supplement for 14 days as observed herein, is indicative of a dietary lipid influence on platelet-vessel wall interactions. The approach of

TABLE 4

Fatty Acid Composition of Phosphatidylinositol in Platelets of Subjects Before Receiving, After Receiving, and After Terminating Supplement of Cod-Liver Oil[1]

Fatty acid	Before receiving supplement	After receiving supplement	After terminating supplement
		(Weight % of total)	
16:0	1.3 ± 0.4^{a}	3.1 ± 0.8^{ab}	4.2 ± 0.7^{b}
16:1	0.6 ± 0.2^{a}	1.0 ± 0.5^{a}	1.0 ± 0.2^{a}
18:0	41.3 ± 1.2^{ab}	43.6 ± 1.2^{b}	39.2 ± 0.7^{a}
18:1	19.2 ± 1.6^{a}	16.9 ± 0.5^{ab}	14.2 ± 0.5^{b}
18:2	2.0 ± 0.2^{a}	2.0 ± 0.1^{a}	2.8 ± 0.2^{b}
20:1	0.2 ± 0.1^{a}	0.9 ± 0.1^{b}	0.3 ± 0.1^{a}
20:2	1.1 ± 0.2^{a}	1.1 ± 0.1^{a}	1.4 ± 0.2^{a}
20:3	1.1 ± 0.1^{a}	0.8 ± 0.1^{b}	1.0 ± 0.0^{ab}
20:4	31.4 ± 1.1^{ab}	28.3 ± 1.6^{a}	33.0 ± 0.9^{b}
20:5	<0.1^{a}	0.5 ± 0.1^{b}	<0.1^{a}
22:4	0.5 ± 0.1^{a}	0.6 ± 0.2^{a}	0.3 ± 0.1^{a}

[1]See legend to Table 1.

TABLE 5

Fatty Acid Composition of Sphingomyelin in Platelets of Subjects Before Receiving, After Receiving, and After Terminating Supplement of Cod-Liver Oil[1]

Fatty acid	Before receiving supplement	After receiving supplement	After terminating supplement
		(Weight % of total)	
16:0	23.2 ± 0.7^{a}	24.2 ± 0.9^{a}	25.6 ± 2.2^{a}
16:1	1.3 ± 0.2^{a}	0.4 ± 0.2^{b}	1.0 ± 0.2^{ab}
18:0	4.8 ± 0.4^{a}	5.1 ± 0.2^{a}	5.0 ± 0.4^{a}
18:1	2.3 ± 0.3^{a}	2.0 ± 0.6^{a}	1.9 ± 0.2^{a}
20:0	6.5 ± 0.3^{a}	5.1 ± 0.2^{b}	6.5 ± 0.3^{a}
20:1	0.6 ± 0.1^{a}	0.7 ± 0.2^{a}	0.4 ± 0.2^{a}
22:0	24.2 ± 0.6^{a}	19.8 ± 0.9^{b}	23.3 ± 0.8^{a}
22:1	3.5 ± 0.2^{a}	2.8 ± 0.2^{a}	3.1 ± 0.7^{a}
23:0	3.7 ± 0.2^{a}	3.6 ± 0.2^{a}	3.4 ± 0.4^{a}
24:0	10.8 ± 0.6^{a}	10.4 ± 0.6^{a}	11.0 ± 0.8^{a}
24:1	17.8 ± 0.5^{a}	24.8 ± 0.7^{b}	17.2 ± 1.2^{a}

[1]See legend to Table 1.

these hematological parameters towards basal (pre-supplement) values as seen 14 days after terminating supplementation is supportive of this concept. This conclusion is consistent with that by Dyerberg (33) and other investigators who have reported increases in Ivy bleeding times (3,8,11) by 11-48% and decreases in platelet aggregation induced by collagen and/or ADP as studied using platelet-rich plasma (3, 8-11) when human volunteers consumed fish, fish oil or an EPA-ethyl ester concentrate made from cod-liver oil. The in vitro addition of EPA to platelet-rich plasma has been reported to inhibit thrombin-induced platelet aggregation (34).

In agreement with the work of Goodnight et al. (9), who tested salmon oil, the ingestion of the cod-liver oil supplement did not significantly alter protein levels/10^{9} platelets. The level of platelet phospholipid, not reported in the previous work (9), also was not significantly changed as neither was platelet cholesterol.

It was of considerable interest in the present study to determine the influence of receiving a cod-liver oil supplement on the level and fatty acid compositions of individual phospholipids in human platelets. The recovery of these parameters was also monitored following termination of the supplement with each subject serving as their own control. Although not measured in previous work (3,4,8-11), the relative abundance of the individual phospholipids was found not to be significantly altered by ingesting the fish oil supplement (Figure 3). The fatty acid compositions of the individual phospholipids from the platelets isolated just prior to supplementation (Tables 1-5) were in general agreement with previous findings on

platelets from normal subjects (29,30,35). The marked increases in the percentage of EPA (20:5ω3) and docosahexaenoic acid (22:6ω3), and decreases in arachidonic acid (20:4ω6), in PC, PE, PS, and PI with cod-liver oil supplementation (Tables 1-4) have been reported previously for the total platelet phospholipid of subjects ingesting a diet enriched in fish or supplemented with fish oil (4,8). All four phospholipids also exhibited an increase in 20:1 with supplementation (Tables 1-4) as found for total phospholipid (8). Even though SPH did not incorporate any significant quantity of EPA (Table 5), the level of 24:1 increased significantly with supplementation, in keeping with the finding of Brox et al. (10) that lower levels of this fatty acid were present in platelet SPH from subjects receiving a cod-liver oil rather than a corn oil supplement.

An increased ratio of EPA/arachidonate in platelet phospholipid has been centrally implicated in the mechanisms proposed to explain the dampening effect of dietary fish oil on platelet aggregation (33). The present work indicates considerable heterogeneity in the EPA/arachidonate ratio amongst the individual phospholipids (Tables 1-4) with mean values of 0.30, 0.14, 0.044, and 0.018 present in PC, PE, PS, and PI, respectively, following supplementation. These patterns are of interest because the response of PI to dietary fish oil has not been examined in any previous study, even though this phospholipid and its metabolic turnover have been implicated in platelet activation and the release of arachidonic acid for conversion to prostaglandins including thromboxane A_2 (12-16). From the present results, it can be calculated that the mean EPA concentration following cod-liver oil supplementation for 14 days in the PC, PE, PS, and PI was 2.92, 2.09, 0.18, and 0.08 $\mu g/10^9$ platelets, respectively, which accounted for 55.4, 39.7, 3.4, and 1.5% of the total EPA in platelet phospholipid. This low proportional association with PI may reflect a rapid turnover (entry and release) of EPA in this phospholipid. In support of this concept, [1-^{14}C]EPA has been found to be readily incorporated into PI in in vitro studies with isolated platelet suspensions and PRP (36).

The fact that the alterations and recovery of the fatty acid compositions of the individual phospholipids with supplementation and after terminating supplementation, respectively, correlate with the bleeding time and platelet aggregation responses supports a key relationship of these fatty acid changes to the hematological parameters and platelet-vessel wall interactions. The specific phospholipid(s) and their component molecular species responsible for mediating the anti-aggregatory effect of dietary fish oil remain to be investigated.

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REFERENCES

1. Dyerberg, J.; Bang, H.O.; Stoffersen, E.; Moncada, S., and Vane, J.R. (1978) Lancet 2, 117.
2. Dyerberg, J., and Bang, H.O. (1979) Lancet 2, 433-435.
3. Sanders, T.A.B.; Naismith, D.J.; Haines, A.P., and Vickers, M. (1980) Lancet 2, 1189.
4. Siess, W.; Scherer, B.; Bohlig, B.; Roth, P.; Kurgmann, I., and Weber, P.C. (1980) Lancet 1, 441-444.
5. Black, K.L.; Culp, B.; Madison, D.; Randall, O.S., and Lands, W.E.M. (1979) Prostaglandins and Medicine 3, 257-268.
6. Culp, B.R.; Lands, W.E.M.; Lucchesi, B.R.; Pitt, B., and Romson, J. (1980) Prostaglandins 20, 1021-1029.
7. Hay, C.R.M.; Durber, A.P., and Saynor, R. (1982) Lancet 2, 1269-1272.
8. Sanders, T.A.B.; Vickers, M., and Haines, A.P. (1981) Clin. Sci. 61, 317-324.
9. Goodnight, S.H. Jr.; Harris, W.S., and Connor, W.E. (1981) Blood 58, 880-885.
10. Brox, J.H.; Killie, J.E.; Gunnes, S., and Nordoy, A. (1981) Thrombosis and Haemostasis 46, 604-611.
11. Thorngren, M., and Gustafson, A. (1981) Lancet 2, 1190-1193.
12. Rittenhouse-Simmons, S. (1979) J. Clin. Invest. 63, 580-587.
13. Lapetina, E.G., and Cuatrecasas, P. (1979) Biochim. Biophys. Acta 573, 394-402.
14. Bell, R.L., and Majerus, P.W. (1980) J. Biol. Chem. 255, 1790-1792.
15. Broekman, M.J.; Ward, J.W., and Marcus, A.J. (1980) J. Clin. Invest. 66, 275-283.
16. Kawahara, Y.; Takai, Y.; Minakuchi, R.; Sano, K., and Nishizuka, Y. (1980) Biochem. Biophys. Res. Commun. 97, 309-317.
17. Bills, T.K.; Smith, J.B., and Silver, M.J. (1977) J. Clin. Invest. 60, 1-6.
18. McKean, M.L.; Smith, J.B., and Silver, M.J. (1981) J. Biol. Chem. 256, 1522-1524.
19. Broekman, M.J.; Ward, J.W., and Marcus, A.J. (1981) J. Biol. Chem. 256, 8271-8274.
20. Miale, J.B. (1967) Laboratory Medicine Hematology. 3rd ed. C.V. Mosby Co., St. Louis, 1192.
21. Lowry, O.H.; Rosebrough, N.J.; Farr, A.L., and Randal, R.J. (1951) J. Biol. Chem. 193, 265-275.
22. Born, G.V.R. (1962) Nature 149, 927-929.
23. Folch, J.; Lees, M., and Sloane, S.G.H. (1957) J. Biol. Chem. 226, 496-509.
24. Bhandaru, R.R.; Srinivasan, S.R.; Pargaonkar, P.S., and Berenson, G.S. (1977) Lipids 12, 1078-1080.
25. Skipski, V.P.; Peterson, R.F., and Barclay, M. (1964) Biochem. J. 90, 374-378.
26. Chapkin, R.S.; Haberstroh, B.; Liu, T., and Holub, B.J. (1983) J. Lab. Clin. Med. 101, 726-735.
27. Duncan, D.B. (1971) Biometrics 11, 1-42.

28. Shattil, S.J.; Bennett, J.S.; Colman, R.W., and Cooper, R.A. (1977) J. Lab. Clin. Med. 89, 341-353.
29. Cohen, P., and Derksen, A. (1969) Brit. J. Haematol. 17, 359-371.
30. Marcus, A.J.; Ullman, H.L., and Safier, L.B. (1969) J. Lipid Res. 10, 108-114.
31. Shastri, K.M.; Carvalho, A.C.A., and Lees, R.S. (1980) J. Lipid Res. 21, 467-472.
32. Owen, J.S.; Hutton, R.A.; Day, R.C.; Bruckdorfer, K.R., and McIntyre, N. (1981) J. Lipid Res. 22, 423-430.
33. Dyerberg, J. (1981) Phil. Tran. R. Soc. Lond. 294, 373-381.
34. Jakubowski, J.A., and Ardlie, N.G. (1979) Thromb. Res. 16, 205-217.
35. Mahadevappa, V.G., and Holub, B.J. (1982) Biochim. Biophys. Acta 713, 73-79, 1982.
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LETTER TO THE EDITOR

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In Figure 5B, p. 799 of 1983 Lipids Vol. 18, Grummer, R.R. et al. illustrate two arcs for the high density lipoprotein fraction of bovine lipoproteins, and yet do not comment upon this phenomenon. It seems possible that these two arcs represent dissociation of apolipoproteins A-I and A-II brought about by freezing and thawing before electrophoresis, or the presence of nonesterified fatty acids (NEFAs) in the bloodstream (Muckle T.J. 1976 Clin. Chem. Acta 73; 57-61 and 1976 Biochem. Med. 15; 17-21). The electrophoretic mobility of albumin can also be affected by the presence of NEFAs as well as other substances (Muckle T.J. 1978 J. Chromatogr. (Biomedical Applications) 146, 77-84), and minor contamination of the HDL fraction by such modified albumin can also produce this phenomenon.

T.J. MUCKLE, M.D., F.R.C.P. (C),
Director of Laboratories,
Chedoke Hospital Division.
Chedoke-McMaster Hospitals,
P.O. Box 2000, Station A,
Hamilton, Ontario, Canada L8N 3Z5

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28. Shattil, S.J.; Bennett, J.S.; Colman, R.W., and Cooper, R.A. (1977) J. Lab. Clin. Med. 89, 341-353.
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T.J. MUCKLE, M.D., F.R.C.P. (C),
Director of Laboratories,
Chedoke Hospital Division.
Chedoke-McMaster Hospitals,
P.O. Box 2000, Station A,
Hamilton, Ontario, Canada L8N 3Z5

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METHODS

Analysis of Molluscan Sterols: Colorimetric Methods

MARY L. SWIFT, *Department of Biochemistry, College of Medicine, Howard University, Washington, D.C. 20059*

ABSTRACT

The wide variety of sterols normally found in extracts of bivalve molluscs leads to high variability in analytical data obtained with colorimetric (chole)sterol methods. Total sterol levels in oyster (*Crassostrea virginica*) extracts were determined using the Liebermann-Burchard reagent, an acid-$FeCl_3$ reagent and a cholesterol oxidase procedure. The data from the latter two agreed to within 5.4% and yielded about 30% higher estimates of sterol content than the Liebermann-Burchard test. Gas-liquid chromatographic data also are compared.

Several pure sterols, selected because of their presence in oyster sterol fractions or because of their structural similarities to such sterols, were examined using each of the three procedures. Sterols, differing from cholesterol only with regard to the side chain, reacted 80-102% as well as cholesterol with the acid-$FeCl_3$ reagent and cholesterol oxidase. The Liebermann-Burchard reaction was more specific for cholesterol. The colorimetric cholesterol oxidase method is recommended for the estimation of total molluscan sterol content.

Lipids 19:625-630, 1984.

The simplicity, efficiency and inexpensiveness of quantitative colorimetric sterol methods help to maintain their popularity. However, the problems encountered with colorimetric cholesterol/sterol analysis on extracts of molluscs have been recognized since 1937 (1). Problems of reproducibility, correct chromogen production and accuracy may be traced to the wide variety of sterols normally found in these animals (2,3). For example, tissues of the oyster, *Crassostrea virginica* Gmelin, may contain up to 35 sterols (4). The steroid nuclei of 90.4% of the identified sterols are identical to those of cholesterol. Of the remaining sterols, 7% differed from cholesterol in the steroid nucleus and in some cases in the side chains as well. Similar varieties of sterols have been identified in other marine fauna: gastropods, cephalopods, crustacea and vertebrate fishes (2,5,6, 7,8).

Recent reports addressing methodological difficulties encountered in the analysis of invertebrate sterols have been either modifications of classic techniques (2,3) or comparisons of results obtained by two or more procedures (9,10,11). The present study evaluates three methods for the estimation of sterols: Liebermann-Burchard (12), acid-$FeCl_3$ (13), and cholesterol oxidase (14). First, the results from analyses of free sterol content in oysters are compared. In addition, several pure sterols selected because of their presence in molluscan sterol fractions or because of their structural similarities to such sterols, were examined using each of the three techniques. After reaction of the selected sterols, the visible spectra of the developed chromogens and the relative absorbance were measured.

MATERIALS AND METHODS

Oysters, *Crassostrea virginica* Gmelin, were obtained from a retail outlet (Capt. White and Sons Seafood, Washington, D.C. 20024). After removal of the shells and separation of tissues, lipids were extracted according to Folch et al. (15). Free sterols were isolated by silicic acid thin-layer chromatography using n-hexane/diethyl ether/glacial acetic acid (70:30:1, v/v/v) for development (16). Mobility of standard cholesterol was used to identify the free sterol bands. The silicic acid containing the sterols was scraped into a small glass syringe and eluted with 2:1 (v/v) chloroform/methanol. The chloroform/methanol was evaporated from aliquots taken for analysis. The sterols were redissolved in solvents appropriate for the selected analytical procedure.

Sterols, free or as digitonides (17), were estimated using three methods. A modified Liebermann-Burchard reaction (12) was used. The sterols, dissolved in 1 ml of glacial acetic acid, were treated with 2 ml of an ice cold 20:1 (v/v) mixture of acetic anhydride/concentrated H_2SO_4. The color was developed in the dark for 30 minutes and read at 625 nm. Second, the sterols were estimated using an acid-$FeCl_3$ reagent (13). Sterols, in 2 ml of glacial acetic acid, are treated with 2 ml of the acid-

$FeCl_3$ reagent. This reagent consists of a 2.5% solution $FeCl_3 \cdot 6H_2O$ in 85% H_3PO_4, which is diluted 1:10 with concentrated H_2SO_4 just before use. The color is allowed to develop for 30 minutes and read at 560 nm. Last, free sterols were assayed using cholesterol oxidase (14).

In addition, aliquots of oyster sterols were analyzed by gas liquid chromatography to confirm that the samples contained a mixture of sterols. Free sterols were separated by gas-liquid chromatography on 1.5% SE-30 (18) using 5α-cholestane as an internal standard. Steryl acetates were separated similarly on 3% SE-30 (19).

Authentic samples of 24-methylcholest-5-en-3β-ol and 24-ethylcholest-5-en-3β-ol were obtained from Applied Science (State College, Pennsylvania, 16801). Cholesterol, 24-ethylcholest-5-22-dien-3β-ol, 5α-cholestane, 5α-cholestan-3β-ol, cholest-5,7-dien-3β-ol, ergosta-5,7,22-trien-3β-ol and 3-keto-24-ethylcholest-4,22-diene were purchased from Sigma Chemical Co. (St. Louis, Missouri 63178). Cholesterol oxidase, S.V.R. was obtained from Calbiochem (LaJolla, California 92037). Digitonin, analytical grade reagents and solvents were from Fisher Scientific Co. (Silver Spring, Maryland 20910). Absorption spectra were determined with a Beckman Model 25 UV/VIS Spectrophotometer (Beckman Instruments, Inc. Fullerton, California 92634). A Shimadzu Seisakusho Model GS-5A gas liquid chromatograph (American Instrument Co., Silver Spring, Maryland 20910) was used.

RESULTS AND DISCUSSION

The results obtained from the analyses of a series of oyster free sterol fractions, using each of the three methods are shown in Table 1. The values given by the Liebermann-Burchard reaction are on average 36% and 29% lower than those obtained using the acid-$FeCl_3$ and cholesterol oxidase methods, respectively. Values estimated using the acid-$FeCl_3$ and cholesterol oxidase techniques agreed, on average, to within 5.4% This is consistent with data previously reported (8,9).

GLC analysis of these oyster samples revealed seven major sterol constituents. Chromatography of oyster free sterols in 1.5% SE-30 yielded six peaks (Fig. 1). Tentative identification was made by comparing retention ratios related to 5α-cholestane (18). Peaks 2 through 6 had retention ratios equal to those reported for cholesta-5,22-dien-3β-ol, cholesterol, cholesta-5,24-dien-3β-ol or 24-methylcholesta-5,22-dien-3β-ol, 24-methylcholesta-5-en-3β-ol and 24-ethylcholesta-5-en-3β-ol, respectively. Note that 24-methylcholest-5-en-3β-ol and 24-methylene cholesterol co-chromatograph on this column. Oyster steryl acetates separated by chromatography on 3% SE-30 yielded eight peaks (Fig. 2). Peaks 2 through 8 had retention

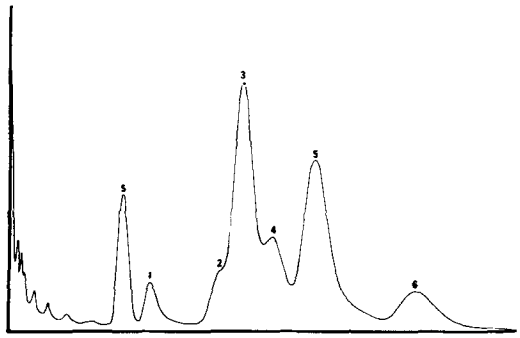

FIG. 1. Chromatogram obtained when oyster free sterols are separated on 1.5% SE-30. Internal standard, peak S, is 5α-cholestane. See text for peak identification.

TABLE 1

Oyster Free Sterol Values Obtained Using Three Methods

Method / Sample	Liebermann-Burchard	Acid-$FeCl_3$	Cholesterol oxidase
	(mg/g wet wt. tissue ± S.D.)		
Group I			
Whole animal	0.69 ± 0.01 (2)	1.06 ± 0.07 (3)	1.03 ± 0.11 (8)
Mantle & gills	0.88 ± 0.01 (3)	1.22 ± 0.09 (3)	1.15 ± 0.04 (3)
Dig. divert. & gonads	1.01 ± 0.03 (2)	1.13 ± 0.10 (3)	1.20 ± 0.10 (3)
Group II			
Whole animal	0.72 ± 0.03 (4)	1.01 ± 0.11 (2)	0.81 ± 0.004 (3)
Mantle & gills	0.84 ± 0.07 (2)	1.16 ± 0.02 (2)	1.14 ± 0.05 (4)
Dig. divert. & gonads	1.01 ± 0.02 (4)	1.35 ± 0.04 (2)	1.31 ± 0.03 (2)

Values in parentheses indicate number of replicate determinations.

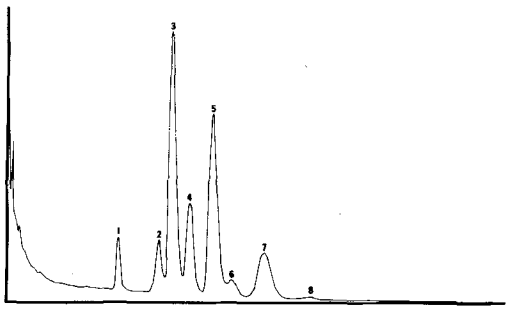

FIG. 2. Chromatogram obtained when oyster steryl acetates are separated on 3% SE-30. See text for peak identification.

ratios, relative to cholesterol, equal to those reported (19) for cholesta-5,22-dien-3β-ol, cholesterol, 24-methylcholesta-5,22-dien-3β-ol, 24-methylene cholesterol, 24-methylcholesta-5-en-3β-ol, 24-ethylcholesta-5,7,22-trien-3β-ol and 24-ethylcholesta-5,7-dien-3β-ol respectively. Peak 1 on both chromatograms could not be identified. From these data and the literature (4,8,20) it may be concluded that the major oyster sterols are: cholesterol, 36-40%, 24-methylene cholesterol, 13-26%, 24-methylcholesta-5,22-dien-3β-ol, 16%, cholesta-5,22-dien-3β-ol, 6-10%, 24-ethylcholesta-5-en-3β-ol, 4% 24-methylcholesta-5-en-3β-ol, 4% and 24-ethylcholesta-5,22-dien-3β-ol, 2%. Recently it has been reported that oysters contain sterols with modified ring systems. Concentrations of $\Delta^{5,7}$ sterols in oysters are reported as totalling 7% of total sterols in *C. virginica* (21) and 16% of total sterols in *Crassostrea gigas* (2). In *C. virginica* cholesta-5,7-dien-3β-ol and ergosta-5,7,22-trien-3β-ol predominate (21).

This variety of sterols found in oyster extracts should raise questions regarding the specificity and degree of reaction of each reagent with the various structural analogues of cholesterol present in these samples. The relative absorbance of the chromophores developed, after reaction of the selected sterols with the Liebermann-Burchard and acid-$FeCl_3$ reagents, is shown in Table 2. Any structural difference among the sterols resulted in a change in the chromogen produced with the Liebermann-Burchard reagent. Of the sterols in Table 2, only 24-ethylcholest-5-en-3β-ol and the $\Delta^{5,7}$ sterols have been compared to cholesterol previously. In all studies, 24-ethylcholest-5-en-3β-ol had a lower molar absorption coefficient at 625 nm than cholesterol (22,23) and the $\Delta^{5,7}$ sterols yielded 130% to 250% as much color as cholesterol after treatment with the Liebermann-Burchard reagent (2,14,22,24,25). Absorbance of the chromogen produced upon reaction with the acid-$FeCl_3$ reagent is within a narrower range when the nucleus is the same as that of cholesterol.

Inspection of the absorption spectra produced upon reaction of the sterols with these two reagents confirms these results. Spectra obtained after reaction of the sterols with the Liebermann-Burchard reagent were dissimilar with regard to absorbance (Table 2) and wavelength maxima (Table 3). On the other hand, spectra of sterols with identical steroid nuclei treated with the acid-$FeCl_3$ reagent had constant wavelength maxima of 560 nm. Any alteration in the steroid nucleus caused marked changes in the spectra.

Spectra obtained after reaction of cholesterol and oyster sterols with the Liebermann-Burchard and acid-$FeCl_3$ reagents are displayed

TABLE 2

Reactivity of Selected Sterols With the Liebermann-Burchard and Acid-$FeCl_3$ Reagents

Sterol	Relative absorbance		Structural difference
	Liebermann-Burchard[a]	Acid-$FeCl_3$[b]	
Cholesterol	100	100	–
24-Methylcholest-5-en-3β-ol	79.5	107	Side chain
24-Ethylcholest-5-en-3β-ol	53.6	85.1	Side chain
24-Ethylcholest-5,22-dien-3β-ol	67.3	95.8	Side chain
5α-Cholestan-3β-ol	2.73	6.55	Ring
Cholest-5,7-dien-3β-ol	218	26.2	Ring
Ergosta-5,7,22-trien-3β-ol	242	37.9	Ring & side chain
3-Keto-24-ethylcholest-4,22-diene	7.27	9.83	Ring & side chain
5α-Cholestane	0	0	Ring
Oyster sterols[c]	81	98	Ring & side chain

[a]Concentration of 2.0 μM, measured at 625 nm.
[b]Concentration of 0.4 μM, measured at 560 nm.
[c]Calculated based on percentage of sterols present as determined by GLC on 3% SE-30.

TABLE 3

Spectral Characteristics of Sterols after Treatment with the Liebermann-Burchard and Acid-$FeCl_3$ Reagents

Sterol	Major absorbance bands		Structural difference
	Liebermann-Burchard	Acid-$FeCl_3$	
	(λ nm)		
Cholesterol	630	560	–
24-Methylcholest-5-en-3β-ol	630	560	Side chain
24-Ethylcholest-5-en-3β-ol	677	560	Side chain
24-Ethylcholest-5,22-dien-3β-ol	693	560	Side chain
5α-Cholestan-3β-ol	–	343,450,553,510	Ring
Cholest-5,7-dien 3β-ol	660	385,490	Ring
Ergosta-5,7,22-trien-3β-ol	665	385,465	Ring & side chain
3-Keto-24-ethylcholest-4,22-diene	694	383,465	Ring & side chain
5α-Cholestane	–	–	Ring
Oyster sterols	630,675	560	Ring & side chain

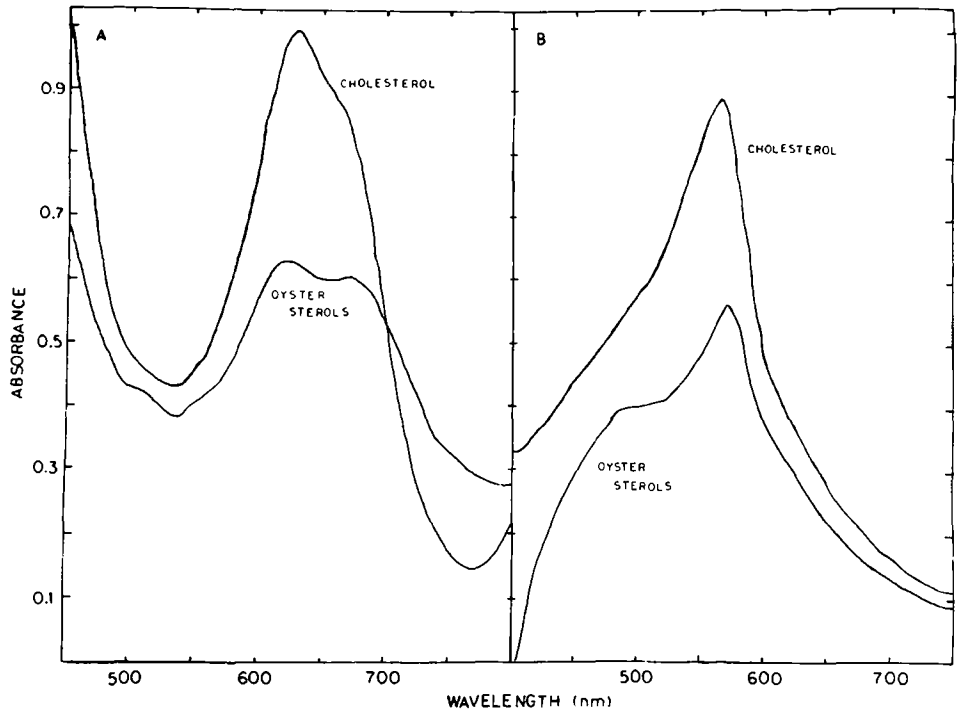

FIG. 3. Shown are the spectra obtained after reaction of cholesterol and oyster sterols with: A) the Liebermann-Burchard reagent; B) the acid-$FeCl_3$ reagent.

in Fig. 3. The chromogen developed by reaction of oyster sterols with the acid-$FeCl_3$ reagent appears quite similar to that developed using cholesterol.

The use of digitonides in these experiments produced results which were nearly identical to those reported above.

Relative reactivity of the selected sterols with cholesterol oxidase is shown in Table 4. The sterols closely related structurally to cholesterol reacted almost as well as cholesterol with the enzyme. Reaction of each sterol with cholesterol oxidase was further examined over a concentration range of 0.02-0.2 μM. A linear response was observed over a concentration range of 0.02 to 0.2 μM, and the data were fitted to the best straight line by regression analysis. Cholesterol oxidase from *Norcardia*

TABLE 4

Reactivity of Selected Sterols with Cholesterol Oxidase

Sterol	Relative absorbance[a]	Slope due to regression[b]	Structural difference
Cholesterol	100	1.39 ±0.08	–
24-Methylcholest-5-en-3β-ol	86.2	2.12 ±0.15	Side chain
24-Ethylcholest-5-en-3β-ol	102	2.85 ±0.04	Side chain
24-Ethylcholest-5,22-dien-3β-ol	99.6	2.29 ±0.10	Side chain
5α-Cholestan-3β-ol	89.8	2.19 ±0.10	Ring
Cholest-5,7-dien-3β-ol	62.5	0.954 ±0.099	Ring
Ergosta-5,7,22-trien-3β-ol	12.7	0.157 ±0.044	Ring & side chain
3-Keto-24-ethylcholest-4,22-diene	2.47	–	Ring & side chain
5α-Cholestane	17.3	–	Ring
Oyster sterols[c]	97	–	Ring & side chain

[a]Concentration of 0.1 μM.
[b]Concentration range of 0.02-0.2 μM.
[c]Calculated based on percentage of sterols present as determined by GLC on 3% SE-30.

erythropolis has been shown to react with all sterols used in this study (26). However, in many instances the degree of reactivity noted here is higher than that previously reported (23,26,27).

In summary, the Liebermann-Burchard reagent is not recommended for the analysis of molluscan sterols. This reagent develops specific chromogens with individual sterols that differ significantly from those developed with cholesterol. Oyster sterols treated with the Liebermann-Burchard reagent develop a chromogen which is dissimilar to the one produced by the reactions with cholesterol.

Even though the acid-$FeCl_3$ and cholesterol oxidase methods gave comparable results, the latter procedure is preferred. The enzyme reacts well with all of the types of sterols that occur in molluscan samples. The enzymatic analysis yields linear results over the concentration range at which the various sterols may be found in molluscan samples. Further, possibilities for the formation of interfering chromogens are minimized since the assay depends upon the production of hydrogen peroxide and not the sterol degradation product (14).

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REFERENCES

1. Bergmann, W. (1937) J. Biol. Chem. 119, 171-176.
2. Gordon, D.T., (1982) J. Amer. Oil Chem. Soc. 59, 536-545.
3. Kritchevsky, D., and DeHoff, J.L. (1978) J. Food Sci. 43, 1786-1787.
4. Teshima, S., Patterson, G.W., and Dutky, S.R. (1980) Lipids 15, 1004-1011.
5. Idler, D.R.; Khalil, M.W.; Brooks, C.J.W.; Edmonds, C.G., and Gilbert, J.D. (1978) Comp. Biochem. Physiol. 59B, 163-168.
6. Takagi, T.; Sakai, A,; Hayashi, K., and Itahashi, Y. (1979) Lipids 14, 5-8.
7. Sica, D. (1980) Comp. Biochem. Physiol. 65B, 407-410.
8. Kritchevsky, D.; Tepper, S.A.; Ditullo, N.W., and Holmes, W.L. (1967) J. Food Sci. 32, 64-66.
9. Fukui, I.; Kushiro, H.; Arisue, K.; Yamaguchi, Y.; Ogawa, Z.; Hayashi, C., and Yamamura, Y. (1977) Atherosclerosis (Ber) 4, 583-588.
10. Kushiro, H.; Nakamoto, J.; Fukui, I.; Ogawa, Z.; Yamaguchi, Y.; Arisue, K.; Hayashi, C., and Yamamura, Y. (1982) Asian Med. J. 25, 69-74.
11. Fukui, I.; Kushiro, H.; Nakamoto, J.; Arisue, K.; Yamaguchi, Y.; Ogawa, A.; Hayashi, C., and Yamamura, Y. (1982) Inter. Congr. Ser. – Excepta Med. 549, 290-295.
12. Abell, L.L.; Levy, B.B.; Brodie, B.B., and Kendall, F.E. (1952) J. Biol. Chem. 195, 357-366.
13. Rosenthal, H.L.; Pfluke, M.L., and Buscaglia, S. (1957) J. Lab. & Clin. Med. 50, 318-322.
14. Allain, C.C.; Poon, L.S.; Chan, C.S.G.; Richmond, W., and Fu, P.C. (1974) Clin. Chem. 20, 470-475.
15. Folch, J.; Lees, M., and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497-509.
16. Malins, D.C., and Mangold, H.K. (1960) J. Am. Oil Chem. Soc. 37, 576-584.
17. Sperry, W.M., and Webb, M. (1950) J. Biol. Chem. 187, 97-106.
18. Ikekawa, N.; Watanuki, R.; Tsuda, K., and Sakai, K., (1968) Anal. Chem. 40, 1139-1141.
19. Patterson, G.W. (1971) Anal. Chem. 43, 1165-1170.
20. Berenberg, C.J., and Patterson, G.W. (1981) Lipids 16, 276-278.
21. Teshima, S., and Patterson, G.W. (1981) Comp. Biochem. Physiol. 68B, 177-181.
22. Haust, H.L., and Beveridge, J.M.R. (1964) Can. J. Biochem. 42, 265-275.
23. Stadtman, T.C.; Cherkes, A., and Anfinsen, C.A. (1954) J. Biol. Chem. 206, 511-523.
24. Idler, D.R., and Baumann, C.A. (1953) J. Biol. Chem. 203, 389-396.
25. Moore, P.R., and Baumann, C.A. (1952) J. Biol.

Chem. 195, 615-621.

26. Smith, A.G., and Brooks, C.J.W. (1976) J. Steroid Biochem. 7, 705-713.

27. Richmond, W. (1973) Clin. Chem. 19, 1350-1356.

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Squalene Isolation by HPLC and Quantitative Comparison by HPLC and GLC

J.C. SULPICE* and J. FÉRÉZOU, *Laboratoire de Physiologie de la Nutrition*[1], *Université de Paris-Sud, Bâtiment 447 91 405 Orsay Cedex France*

ABSTRACT

A new procedure is described for isolating and measuring squalene in plasma and in several organs of the rat. The unsaponifiable material was fractionated by normal phase HPLC on a silica gel column using a mobile phase consisting of hexane/propanol-2/water. The eluate was monitored at 215 nm. The squalene in the hydrocarbon fraction thus collected was than quantified on an analytical column eluted with hexane. Squalene concentrations ranging from 3 to 200 μg per ml of plasma or per g of fresh tissue were accurately measured. The results obtained agree with those of the squalene assays carried out by gas chromatography on a packed or capillary column.
Lipids 19:631-635, 1984.

INTRODUCTION

Squalene, which is the last metabolite preceding sterol ring formation in the biosynthetic cholesterol pathway, has both synthetic and dietary origins. The active participation of dietary squalene in cholesterol production already has been demonstrated (1,2). Moreover, cholesterogenesis has been correlated to the plasma squalene level when a low squalene diet is administered (3). Various approaches have thus been proposed to evaluate the rate of cholesterogenesis by measuring the squalene concentration in plasma or by measuring its specific radioactivity after injection of a radioactive precursor (4,5). The present study concerns a rapid method of squalene isolation which can be useful in the investigation of squalene metabolism. High performance liquid chromatography (HPLC), a convenient method for fractionating lipid mixtures, especially sterols (6,8), was used to isolate squalene from cholesterol, methylsterols and sterol precursors by directly using the unsaponifiable material of the plasma and several organs in the rat. This procedure involved a normal phase preparative HPLC on a silica gel column using hexane/propanol-2/water as the mobile phase, with UV squalene detection at 215 nm. The squalene in the hydrocarbon fraction thus obtained was then accurately measured by HPLC on an analytical column using hexane as the mobile phase. The results were compared to conventional gas-chromatographic assays on a packed or capillary column.

MATERIALS AND METHODS

Materials

Standard cholestane and squalene were obtained from Sigma (St. Louis, Missouri), [U-^{3}H]-squalene and [1,2-^{3}H]-cholesterol, from the Service des Molécules Marquées (C.E.A., Saclay, France). n-Hexane, analytical grade, propanol-2, HPLC grade and petroleum ether (boiling range 35-60 C) were purchased from Prolabo (Paris, France). Water was double distilled. All solvents used for elution were filtered through a membrane with 0.5 μm pores (Millipore Intertec, Inc., Bedford, Massachusetts), sonicated and degassed under vacuum every 3 hours.

The animals used for this study were adult male Wistar rats housed in the laboratory and fed a semi-synthetic diet (9). They were killed by intraaortic puncture after pentobarbital anesthesia. Samples of liver, intestine (wall and mucosa), skin, kidney and adipose tissue were collected after the cardiovascular system was washed with physiological saline. Plasma and tissue samples were directly saponified in 2N alcoholic KOH for 2 hours at 80 C. The unsaponifiable material was extracted three times with petroleum ether, washed with ethanol/water (50:50, v/v) and dried by evaporation.

HPLC

Apparatus

HPLC was conducted with a double pump LIREC A 802 apparatus (LIREC, Montgeron, France) equipped with a manual injector and a variable wavelength detector (cell volume 8 μl, optical path 10 mm). Preparative chromatography was performed on a commercial column (Chrompack, 4330 AA Middelburg, The Netherlands) (25 cm long, 1.2 cm I.D.) packed with SI 60-7 silica gel (Merck, Darmstadt, W. Germany) preceded by a precolumn (5 cm long, 2 mm I.D.) packed with HP Pellosil (30-38 μm) (Whatman, Clifton, New Jersey) which served as a filter for column protection.

*To whom correspondence should be addressed.
[1]Equipe de Recherche Associée au C.N.R.S. : ERA 0415.

This system was connected to a time-programmed fraction collector. For squalene measurements, a commercial analytical column (Chrompack) (25 cm long, 4.6 mm I.D.) packed with Si 60-5 silica gel (Merck) was used. Chromatograms were analyzed with an electronic integrator.

PROCEDURES

Preparative HPLC

A fraction of the unsaponifiable material containing 1 to 10 mg of sterols was dissolved in 1 ml hexane and injected onto the column. The mobile phase was an isohydric mixture (10) consisting of hexane/propanol-2/water (98:1.986:0.014, v/v/v), with a flow rate of 5 ml/min. The eluate was monitored at 215 nm. The retention time of squalene was 4 min. When required, squalene collected in the hydrocarbon fraction was purified by a second-step chromatography on the same column using hexane as the mobile phase, with a flow rate of 5 ml/min. Under these conditions, its retention time was 8 min.

HPLC Measurements

The squalene samples collected by preparative HPLC were dissolved in a convenient volume of hexane to a final squalene concentration ranging from 5 to 50 μg/ml. Squalene assays were carried out using hexane as the mobile phase, with a flow rate of 1 ml/min. A calibration curve was established with known injected amounts of squalene ranging from 0.1 to 1.5 μg. Both the standard and samples were injected from the same completely filled 24 μl loop. This volume was accurately measured by injecting a standard solution of [1,2-^{3}H]-cholesterol into the loop and collecting the sample without passing through the column. Each sample injection was followed by two calibrating injections each spaced 2 minutes apart.

GAS CHROMATOGRAPHY

Apparatus

Gas/liquid chromatography was carried out with a Varian 1400 apparatus (Varian Aerograph, Palo Alto, California) equipped with a FID detector. The glass column (1.5 m long, 2 mm I.D.) was packed with 1.5% OV 202 on 100-120 mesh Chromosorb WHP (Chrompack). Its temperature was 280 C, with the detector and injector at 260 and 270 C respectively.

Capillary gas chromatography was conducted with a Carlo Erba 4100 apparatus (Carlo Erba, Milano, Italy) equipped with a Ross needle type injector and a FID detector. The commercial silica column (25 m long) was coated with SE 52 (Carlo Erba). Its temperature was 255 C with the detector and injector at 285 C. The carrier gas was H_2 at a pressure of 0.3 bar. Chromatograms were analyzed with an electronic integrator.

Procedures

The squalene fractions collected after preparative HPLC or squalene samples used for the calibration curve were dissolved in hexane in the presence of a known amount of cholestane as an internal standard. In order to measure minute amounts of squalene, the capillary column was used and squalene concentrations were adjusted from 0.4 to 2 or from 2 to 10 μg per ml of hexane containing 2 or 10 μg of cholestane respectively. Two microliters were injected. Usually, however, the packed column was used and squalene concentrations were adjusted to 10-100 μg/ml of hexane containing 100 μg of cholestane. The injected volume was three microliters.

In order to evaluate the technical squalene losses that could occur during the purification steps (extraction + preparative HPLC), several squalene assays were carried out after adding a fixed amount of cholestane prior to the samples saponification. Since squalene and cholestane were eluted in the same hydrocarbon fraction by preparative HPLC using hexane/propanol-2/water as the mobile phase, their concentration ratio could be measured by GLC both in the unsaponifiable material and in the squalene fraction thus collected. Using this procedure, squalene amounts were corrected for 100% recovery of the internal standard.

RESULTS AND DISCUSSION

Preparative HPLC on a silica gel column in normal phase proved to be a more rapid and efficient method of isolating squalene from the various sterols present in the biological sample's unsaponifiable material (Figure 1 and Table 1) than thin layer chromatography. The low cost of this procedure was due to the possible use of hexane analytical grade in the mobile phase. However, in hexane/propanol-2/water, squalene was collected with the solvent front in the hydrocarbon fraction (Figure 1). A polarity gradient from hexane to hexane/propanol-2/water would have been more appropriate to improve squalene purification. Unfortunately, the higher resolution of this procedure resulted in the loss of internal standards as cholestane or squalane which could be useful for a further squalene assay by GLC (to correct for a 100%

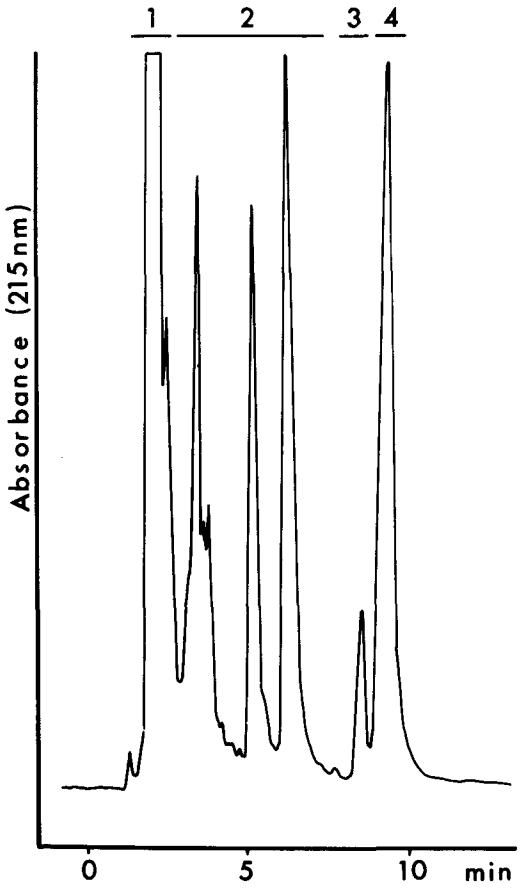

FIG. 1. HPLC separation of squalene, cholesterol and sterol precursors from the unsaponifiable material of rat skin. The column (25 cm long, 1.2 cm I.D.) was packed with Si 60-7 silica gel (Merck). The mobile phase was hexane/propanol-2/water (98:1.986:0.014, v/v/v) and the flow rate 5 ml/min. Fractions 1,2,3 and 4 contained squalene, methylsterols, cholesterol and Δ7-cholesterol, respectively.

recovery of the internal standard). Moreover, such a gradient required appreciable volumes of hexane and several hours to re-equilibrate the column between two injections. Thus, when squalene purification was necessary, especially to measure its specific radioactivity, the preferred method consisted of purifying the squalene fraction first collected in hexane/propanol-2/water as a mobile phase by a subsequent chromatography on the same column preconditioned in hexane.

The quantitative isolation of squalene by preparative HPLC in hexane/propanol-2/water as a mobile phase was verified. More than 95% of a standard [U-^{3}H]-squalene solution was usually recovered in the hydrocarbon fraction, suggesting that squalene retention on the silica gel was negligible. Similarly, when rats received an intravenous injection of radioactive cholesterol precursor, more than 95% of the radioactivity of the unsaponifiable material obtained from the various organs was recovered in four principal fractions called squalene, methylsterols, cholesterol and Δ7-cholesterol (Fig. 1).

TABLE 1

Separation of Squalene, Sterols and Non-Biological Standards

Compound	T_R Retention time in minutes	k' Capacity factor $k' = \frac{T_R - T_0}{T_0}$ [b]
Squalene	4	0.3
Squalane (ST)	4	0.3
Cholestane (ST)	4	0.3
Farnesol (ST)[a]	7.1 and 8.6	1.4 and 1.9
Lanosterol	10	2.3
Cholesterol	17	4.6
Δ7-cholesterol	18.6	5.2

Separation of squalene, sterols and non-biological standards (ST) by HPLC on a Si 60-7 (Merck) silica gel column (25 cm long, 1.2 cm I.D.) using hexane/propanol-2/water (98:1.986:0.014, v/v/v) as the mobile phase (5 ml/min).

[a]Mixture of two isomers.

[b]Retention time of the solvent front (T_0=3 min.).

GAS CHROMATOGRAPHIC SQUALENE ASSAYS

Gas chromatography is the usual method for squalene assays (1-5). In this study, the squalene amounts of the diets and the different organs in the rat were usually measured by using the packed column. However, in rats fed a low squalene diet (5.9 μg/g), the plasma squalene level was too low to be measured accurately. A greater sensitivity and an improved resolution were thus obtained when chromatography was carried out on a capillary column (Fig. 2). The efficiency of this procedure was limited by the purity of solvents used in the chemical treatment of the biological samples.

The main advantage of gas chromatography resided in the possible addition of an internal standard to the biological sample before saponification and collection of the squalene by preparative HPLC using hexane/propanol-2/water as a mobile phase. Using this method, only 5 to 10% of the squalene was estimated to be lost during its purification from several samples of rat plasma, skin and adipose tissue (Table 2).

HPLC Squalene Assays

In the HPLC procedure, common non-bio-

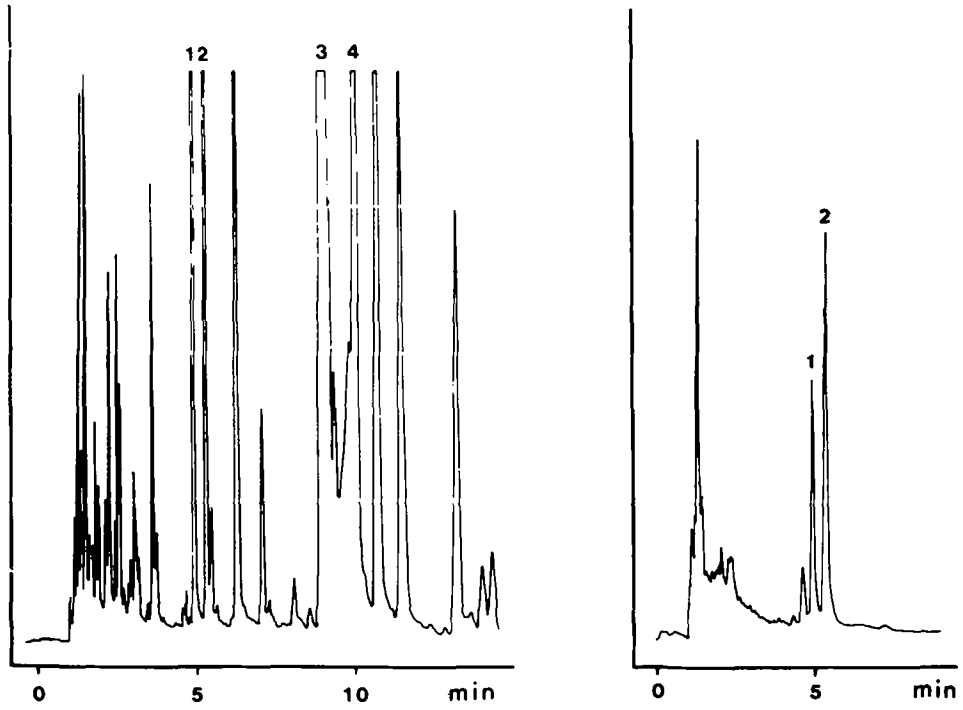

FIG. 2. Capillary gas chromatography of squalene on a silica gel column (25 m long) coated with SE 52. The temperature was 255 C, hydrogen pressure 0.3 bar and detection was flame ionization. A. Analysis of the unsaponifiable material from rat skin. B. Squalene assay in the hydrocarbon fraction prepared by HPLC using hexane/propanol-2/water as the mobile phase, from the unsaponifiable material of rat plasma. The sample was dissolved in 0.5 ml hexane containing 2 μg of cholestane per ml. The injected volume was 2 μl.

Signals 1,2,3 and 4 represent squalene, standard cholestane, cholesterol and Δ7-cholesterol, respectively.

TABLE 2

Squalene Concentration in Plasma and Several Organs

Samples (n)	Plasma (3)	Adipose tissue (4)	Skin (3)
Unsaponifiable material	6.01 ± 0.78	38.3 ± 5.0	129.1 ± 48.5
Squalene fraction	5.30 ± 0.65	36.43 ± 4.93	115.8 ± 44.8

Squalene concentrations (mean value ± standard error of the mean) in plasma (μg/ml) and several organs (μg/g of fresh tissue) from the rat measured by GLC in the unsaponifiable material and in the squalene fraction collected by preparative HPLC. Cholestane was added as an internal standard before saponification of the biological samples.

logical internal standards such as cholestane or squalane generally used in GLC (1-5) were unable to improve squalene assays and correct for its technical losses, since those compounds were not detected at 215 nm. However, squalene was accurately measured in the absence of a convenient internal standard by using a calibration curve obtained from known amount of squalene. In order to improve the precision of the assays, the detector response was calibrated by two injections of standard squalene after each sample injection (Fig. 3). This method was validated by a direct comparison with squalene assays carried out by gas chromatography on a packed or capillary column. The small technical squalene losses

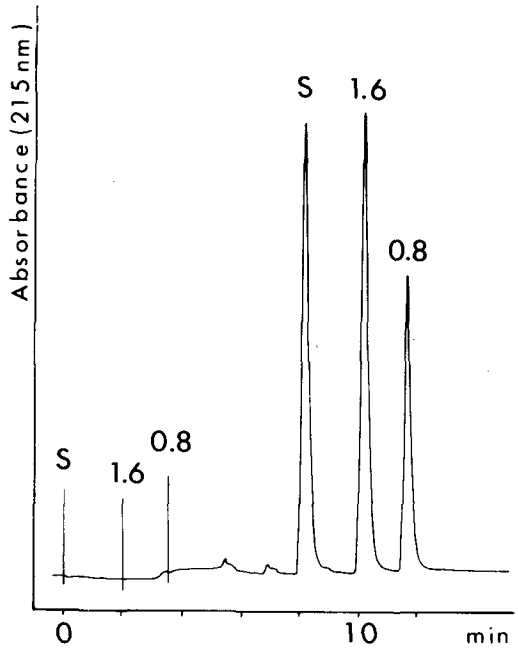

FIG. 3. HPLC assay of the hydrocarbon fraction isolated by preparative HPLC from the unsaponifiable material of rat skin. The column (25 cm long, 4.6 mm I.D.) was packed with Si 60-5 silica gel (Merck) and the mobile phase was hexane with a flow rate of 1 ml/min. The sample injection (S) was followed by two calibrating injections of standard amounts of squalene (1.6 and 0.8 μg). The injected volume was 24 μl.

were not taken into account, and cholestane was added to the squalene fraction prepared by HPLC in hexane/propanol-2/water as the mobile phase. As seen in Table 3, the procedures were similar. Individual squalene concentrations, HPLC versus GLC, expressed in μg/ml of plasma or μg/g of fresh tissue, were related by a regression line (equation $y = 0.953x + 0.9$), with a Pearson correlation coefficient (r) of 0.993.

Either HPLC or GLC can be used for squalene assays. This study has shown that GLC was better for precise squalene concentration measurements, as the use of an internal standard took into account the correction for total squalene recovery during its purification. However, HPLC appeared to be particularly suitable for measuring the specific radioactivity of squalene samples because it enabled purified squalene to be both rapidly isolated and precisely quantified. This method probably could be improved by connecting the HPLC system to an automatic radioactivity detector which is already commercially avalaible.

ACKNOWLEDGMENTS

We wish to thank Pr. J.M. Vernier for providing facilities to carry out the analyses by capillary chromatography.

TABLE 3

Squalene Concentrations in Various Organs, Plasma and Diet of Rats

Samples (n)	Liver (10)	Kidney (4)	Intestine[a] Wall (4)	Intestine[a] Mucosa (2)	Adipose tissue (7)	Skin (9)	Plasma (9)	Diet (5)
HPLC	23.9 ± 2.4	21.3 ± 1.3	8.3 ± 1.5	12.5 ± 3.8	36.4 ± 2.9	100.7 ± 14.5	5.0 ± 0.4	—
GLC	25.4 ± 2.5	23.2 ± 1.2	8.9 ± 0.7	10.0 ± 3.0	36.6 ± 2.8	105.4 ± 18.0	4.7 ± 0.6	5.9 ± 0.8

Squalene concentrations (mean value ± standard error of the mean) in various organs (μg/g of fresh tissue), in the plasma (μg/ml) and the diet (μg/g) of the rats, measured by HPLC and GLC. Measurements were carried out in the squalene fractions collected by preparative HPLC from the unsaponifiable materials.

[a]After its content was removed by washing with physiological saline, the intestine was longitudinally opened and the mucosa was scraped off with a scalpel blade. The wall represents the remaining part of the intestine.

REFERENCES

1. Liu, G.C.K.; Ahrens, E.H.; Schreibman, P.H.; and Crouse, J.R. (1976) J. Lipid Res. 17, 38-45.
2. Tilvis, R.S., and Miettinen, T.A. (1983) Lipids 18, 32-36.
3. Miettinen, T.A. (1969) Life Sc. 8, 713-721.
4. Liu, G.C.K.; Ahrens, E.H.; Schreibman, P.H.; Samuel, P.; Mc Namara, D.J.; and Crouse, J. (1975) Proc. Nat. Acad. Sc. USA 72, 4612-4616.
5. Strandberg, T.E.; Tilvis, R.S.; and Miettinen, T.A. (1981) Scand. J. Gastroent. 16, 801-810.
6. Aitzetmuller, K., and Koch, J. (1978) J. Chromatog. 145, 195-202.
7. Collin, H.; Guiochon, G.; and Slouffi, A. (1979) Anal. Chem. 51, 1661-1666.
8. Hansbury, E., and Scallen, T.J. (1980) J. Lipid Res. 21, 921-929.
9. Serougne, C., and Rukaj, A. (1982) Ann. Nutr. Metab. 27, 386-395.
10. Thomas, J.P.; Brun, A.; and Bounine, J.P. (1977) J. Chromatog. 139, 21-43.

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Mammary Transfer and Metabolism in the Rat of Halogenated Fatty Acids of Halogenated Olive Oil

H.B.S. CONACHER*, R.K. CHADHA, J.F. LAWRENCE, S.M. CHARBONNEAU and **F. BRYCE**, *Bureau of Chemical Safety, Food Directorate, Health Protection Branch, Ottawa, Ontario, Canada. K1A 0L2*

ABSTRACT

To assess possible incorporation of halogenated fatty acids into the neonate via the milk, a 4-day study was carried out in which lactating Wistar rats were orally dosed with either brominated olive oil (BOO) (0.6 g/kg body wt/day) or chlorinated olive oil (COO) (0.4 g/kg body wt/day) for the first 4 days. On days 1-5 inclusive 2 pups per litter were sacrificed and the stomach curd and livers analyzed for halogenated fatty acids by gas liquid chromatography (GLC). On day 5 all dams also were sacrificed and their livers and adipose tissue similarly analyzed. With BOO, brominated fatty acids (bfa) accumulated in both the milk lipids and neonate liver lipids, and appeared to plateau on day 4 at levels of 2% and 5% respectively. In contrast to the BOO in which approximately 100% of the bfa was dibromostearic (DBS), the milk bfa comprised 79% (DBS), 9% dibromopalmitic (DBP) and 12% dibromomyristic (DBM) acids, suggesting maternal metabolism to the shorter chain brominated acids. In the neonate liver lipids the bfa composition was 47% (DBS), 12% (DBP) and 41% (DBM), suggesting either further metabolism in the neonate and/or preferential accumulation of the shorter chain brominated acids. The analysis of maternal tissue indicated very low bfa residues, contrary to previous studies in non-lactating rats. Similar results were obtained with COO.
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INTRODUCTION

In contrast to the transfer of various contaminants such as polychlorinated biphenyls (1), polybrominated biphenyls (2), and halogenated hydrocarbons (3-5) to the neonate via the milk, little has been reported on the mammary transfer of food additives. While it is recognized that food additives have limited toxicity compared to the various contaminants quoted previously it also should be recognized that additives normally are present in foods at much higher levels. Consequently, levels to which the neonate may be exposed in the milk also may be much higher.

Several reports recently have appeared in the literature on the metabolism of brominated fatty acids (bfa) in the rat (6-8). It was shown that both males and females accumulate bfa in the liver, heart and adipose tissue. This communication describes preliminary studies conducted in lactating rats with brominated olive oil (BOO) and with chlorinated olive oil (COO) to assess possible incorporation of halogenated acids into the milk, further absorption into the neonate, and any metabolism occurring in these processes. Brominated vegetable oils presently are permitted as food additives in North America, and chlorinated oils can result from the use of chlorine as a bleaching and maturing agent on some flours (9).

*To whom correspondence should be addressed.

MATERIALS AND METHODS

Brominated olive oil was prepared by bromination of olive oil in cold diethyl ether (10); chlorinated olive oil by chlorination of olive oil in cold carbon tetrachloride in the absence of light (11). The chlorinated oil was demonstrated (12) to contain less than 1 ppm carbon tetrachloride. Solutions of BOO (32%) and COO (20%) in corn oil were prepared; 0.5 ml aliquots were used for dosing by oral intubation.

On the fourth day post partum (day 1 of the study) the litters of lactating Wistar rats (265-306 g, Woodlyn Farms, Guelph, Ontario) were culled to 10 pups. In addition, 2 pups/litter were sacrificed by decapitation (as controls). Six dams were dosed with BOO (0.6 g/kg body wt/day), and 5 dams with COO (0.4 g/kg body wt/day). Previous studies in our laboratories had indicated no apparent visual toxic effects in adult rats dosed at these levels for 10 days. Dosing of dams and sacrificing (2 pups/litter) were continued for the next 3 days. On the fifth day, all dams were sacrificed using ether anesthesia, and the remaining pups by decapitation. The stomach curd and livers of all pups, and the livers and adipose tissue of dams were taken for lipid analysis.

Throughout the study rats were housed in Health Guard System® shoe box cages and permitted free access to laboratory rat chow

(Master Laboratory Cubes, Master Feeds, Toronto, Ontario) and water. The cages were kept in an air conditioned (72 ± 1 F), humidity controlled (50-55%) room with artificial lighting (12 hours light/dark cycle). Necropsies were performed at the same time each day.

Lipid was extracted from the stomach curd, livers and adipose tissue by the method of Bligh and Dyer (13), and converted to methyl esters by refluxing with 2% sulfuric acid in methanol/hexane (5:1). Under these transesterification conditions brominated and chlorinated triglycerides were converted to methyl esters with no loss of bromine (14) or chlorine (15). Methyl pentadecanoate was included as internal standard to permit quantitative measurement of total fatty acid esters.

Gas chromatographic (GC) analyses were carried out on a Varian 2100 chromatograph fitted with two identical 6′ x 2 mm id glass columns packed with 3% OV-3 on 80-100 mesh Chromosorb W, HP. One column was connected to a flame ionization detector (FID), and the other to a Coulson electrolytic conductivity detector operated in the halogen mode. Outputs from the two detectors were displayed on a dual pen recorder as shown in Figures 1, 2 and 5 with the upper and lower tracings representing the Coulson and flame ionization outputs respectively. Columns were temperature programmed from 100-300 C at 10/min, and flow rates were adjusted to permit simultaneous elution of methyl dibromostearate in approximately 16 min. Peak areas were measured by electronic integrator. For quantitation by FID, response factors were calculated for dibromo- and dichlorostearate standards using C_{15} fatty acid as an internal standard. With the Coulson detection system, quantitation was carried out by comparing sample peak areas to external standards of the two halogenated stearates, since C_{15} acid does not produce a response with this detector. Levels of the two metabolites were based on peak areas assuming detector responses are similar to the respective brominated and chlorinated stearates.

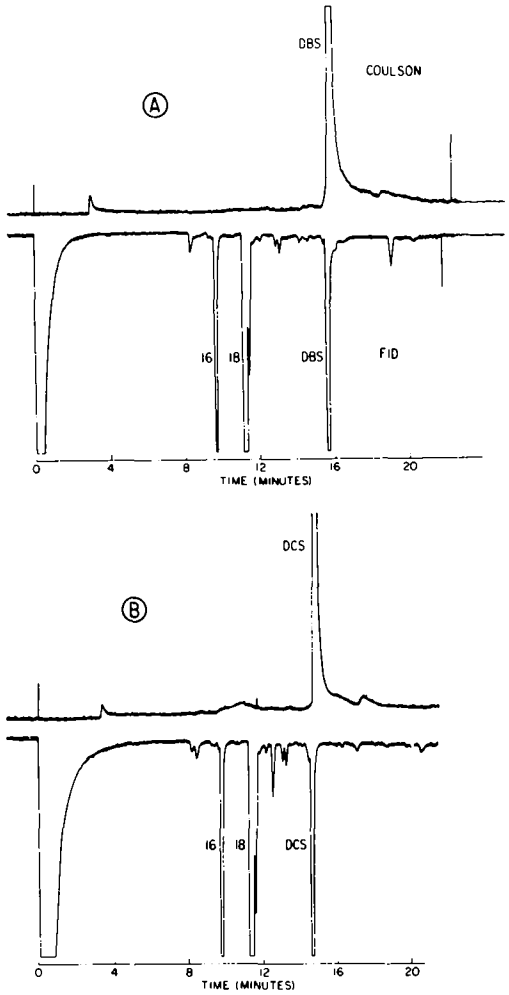

FIG. 1. GLC chromatograms of methyl esters of dosing solution. A – BOO dosing solution methyl esters; B – COO dosing solution methyl esters; DBS – methyl dibromostearate; DCS – methyl dichlorostearate. Numbers adjacent to peaks on FID tracing indicate chain-length of fatty acids in peak immediately to right of number.

RESULTS

Dosing Solutions

Figure 1 shows the GC chromatograms resulting from the transesterification of the dosing solution of BOO (A) and COO (B). The response on the Coulson detector indicated one major peak corresponding to methyl 9,10-dibromostearate in BOO, and the methyl 9,10-dichlorostearate in COO, with no halogenated peaks corresponding to chain-lengths shorter than C_{18} in either case. Peaks corresponding to the tetra-substituted derivatives which would arise from halogenation of linoleic acid present in the original olive oil were not observed under the analytical conditions and were not considered further in this study.

Stomach Curd Lipids

Figure 2 illustrates the fatty acid pattern of the stomach curd lipids prior to dosing (A), and one day after dosing the dams with BOO (B) and with COO (C). Fatty acids ranged in chain-length from approximately C_{10}-C_{22}, and no halogenated acids were present in the curd

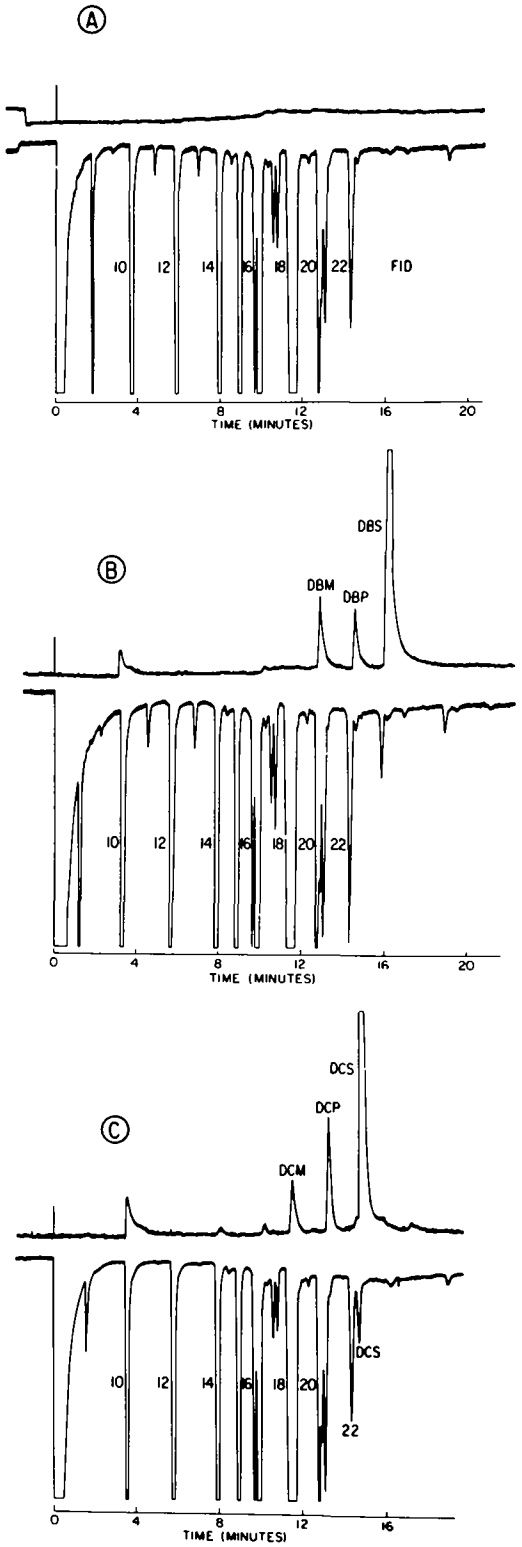

FIG. 2. GLC chromatograms of methyl esters of stomach curd lipids. A – Methyl esters of stomach curd lipids prior to dosing dam; B – Methyl esters of stomach curd lipids one day after dosing dam with BOO; C – Methyl esters of stomach curd lipids one day after dosing dam with COO; DBS – methyl dibromostearate; DBP – methyl dibromopalmitate; DBM – methyl dibromomyristate; DCS – methyl dichlorostearate; DCP – methyl dichloropalmitate; DCM – methyl dichloromyristate. Numbers adjacent to peaks on FID tracing indicate chain-length of fatty acids in peak immediately to right of numbers.

prior to dosing. Brominated (B) and chlorinated (C) acids, however, appeared in the milk lipids one day after dosing. The appearance of halogenated peaks, corresponding to C_{16} and C_{14} di-halogenated acids, indicated some metabolism, presumably β-oxidation, had occurred. It has been shown recently that metabolism of BOO and brominated corn oil in male rats resulted in the formation of C_{14} and C_{16} dibrominated acids (7,8). It also appears that the loss of the 2-carbon fragments stops after C_{14} since no C_{12} acids were reported. Gas chromatography-mass spectrometry was used to identify the metabolites (8).

Shown in Figure 2 is the chromatogram obtained with the Coulson detector for the stomach curd of the neonates of the BOO group. The results are similar to results found earlier (8) for the bfa composition of the livers of rats fed BOO for 4 days. Both results show only the presence of the metabolites, dibromomyristate and dibromopalmitate, in addition to unchanged dibromostearate. The obvious advantages of the Coulson detector in identifying and measuring the halogenated fatty acids are readily apparent from chromatograms B and C. With the flame ionization detector alone, the C_{14} and C_{16} halogenated acids could not be observed since they eluted with, and consequently were obscured by, the more common non-halogenated C_{18}, C_{20} and C_{22} fatty acids.

The total di-halogenated fatty acid contents (C_{18} + C_{16} + C_{14}) of the curd fatty acids for BOO and COO are shown in Figures 3 and 4, respectively. Brominated fatty acids appear to have increased over the dosing period up to a level of approximately 2% where they leveled off. Chlorinated fatty acids appeared to plateau at a slightly lower level of 1.2-1.5%. On a molar basis, the difference between the two becomes negligible.

Although the total halogenated fatty acids increased over the dosing period, the relative percentages of halogenated fatty acids (C_{18}:C_{16}:C_{14}) remained unchanged (Table 1) during this period, and no appreciable differences

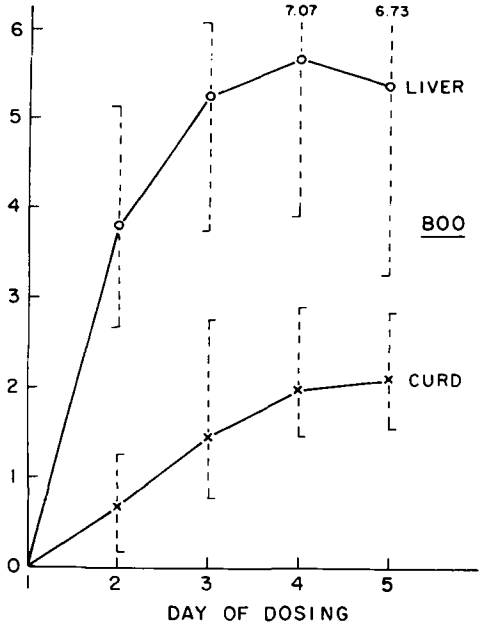

FIG. 3. Brominated fatty acid content of the curd and liver fatty acids over dosing period. Each point shows the mean ± range for 2 rats from each of 6 litters.

were evident between the BOO (79:9:12) and the COO (84:8:8).

The average curd fatty acid contents were 23.4% and 22.8% for BOO and COO, respectively, and remained constant over the dosing period.

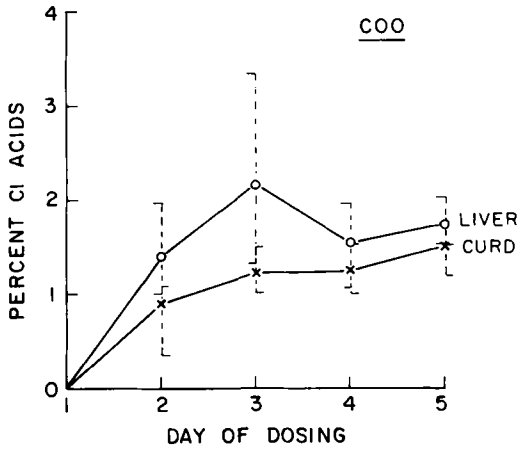

FIG. 4. Chlorinated fatty acid content of the curd and liver fatty acids over dosing period. Each point shows the mean ± range for 2 rats from each of 5 litters.

Neonate Liver Lipids

The fatty acid patterns of the neonate liver lipids prior to dosing (A), and one day after dosing the dams with BOO (B) and with COO (C) and shown in Figure 5. As with the curd, halogenated fatty acids corresponding to the di-halogenated C_{18}, C_{16} and C_{14} acids, which

TABLE 1

Relative Amounts of Different Brominated and of Different Chorinated Acids in Curd and in Neonate Liver Over the Period of Study

	% of Total Halogenated Acids				
	Day 2[a]	Day 3	Day 4	Day 5	Mean
Curd (BOO)					
C18	76 ± 3[b]	78 ± 1	81 ± 4	80 ± 3	79 ± 3
C16	9 ± 1	9 ± 1	9 ± 1	10 ± 2	9 ± 2
C14	15 ± 3	13 ± 3	10 ± 4	10 ± 2	12 ± 4
Curd (COO)					
C18	84 ± 4	85 ± 2	83 ± 3	84 ± 1	84 ± 3
C16	8 ± 2	8 ± 1	8 ± 2	8 ± 2	8 ± 2
C14	8 ± 3	7 ± 3	9 ± 1	8 ± 1	8 ± 2
Liver (BOO)					
C18	41 ± 11	47 ± 4	50 ± 4	49 ± 9	47 ± 8
C16	14 ± 7	11 ± 2	12 ± 2	11 ± 2	12 ± 4
C14	45 ± 11	42 ± 4	38 ± 3	40 ± 8	41 ± 7
Liver (BOO)					
C18	38 ± 7	47 ± 12	50 ± 7	49 ± 5	46 ± 9
C16	8 ± 2	8 ± 3	9 ± 2	11 ± 2	9 ± 3
C14	54 ± 8	45 ± 10	41 ± 6	40 ± 6	45 ± 9

[a]Day 2 is equiv. to 5th day post-partum.
[b]Mean ± std. dev.

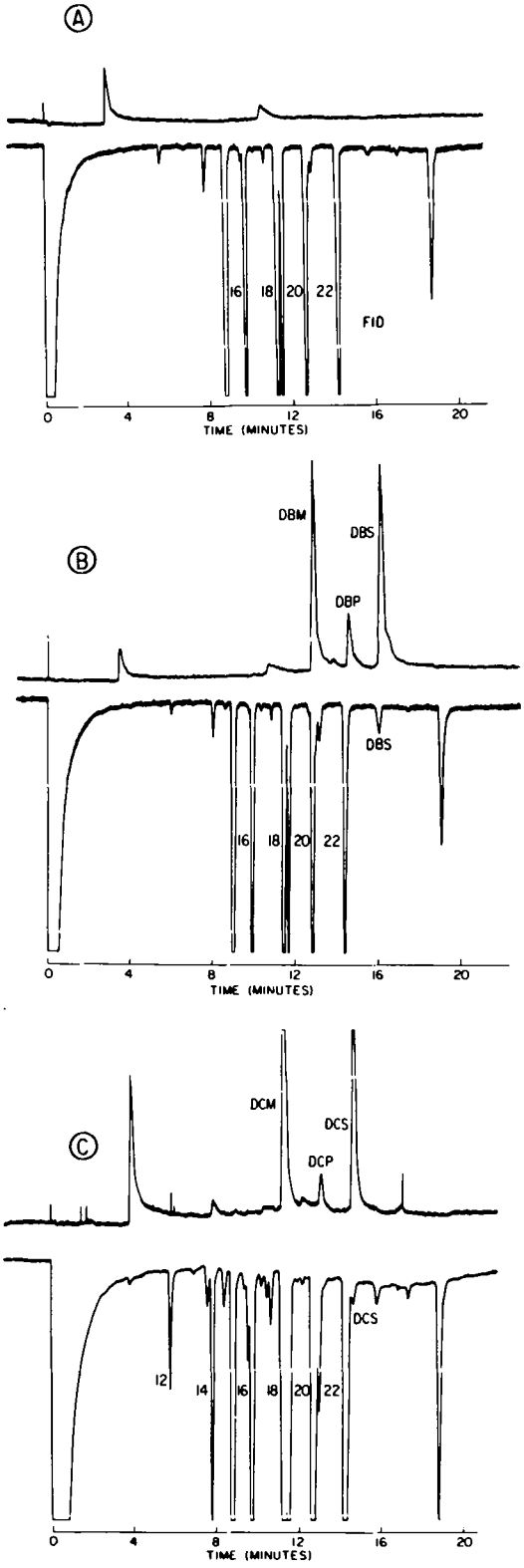

FIG. 5. GLC chromatograms of methyl esters of neonate liver lipids. A – Methyl esters of neonate liver lipids prior to dosing dam; B – Methyl esters of neonate liver lipids one day after dosing dam with BOO; C – Methyl esters of neonate liver lipids one day after dosing dam with COO. Abbreviations as in Figure 2.

were not evident before dosing, appeared one day after dosing. The total di-halogenated fatty acid level in the liver fatty acids increased over the dosing period and appeared to level off at about 5% for BOO (Fig. 3) and at about 2% for COO (Fig. 4). On a molar basis, the halogenated acids from BOO equilibrate at twice the level of those from COO.

Again, the relative percentages of the halogenated acids (excluding possibly the first day after initial dosing) appeared to be unchanged during this period (Table 1) with no appreciable differences between the BOO (47:12:41) and the COO (46:9:45). However, these relative percentages were considerably different from those observed in the milk lipids.

Dam Adipose Tissue and Liver Lipids

Only trace levels (<0.1%) of halogenated lipids were found in the adipose tissue and liver lipids of the dams in the present study. This is in contrast to the higher levels found in the neonate liver lipids and to the higher levels (> 1%) found by us previously (8,15) in the adipose tissue and liver lipids of non-lactating adult rats.

DISCUSSION

The results of this study in which lactating rats were dosed for 4 days with high levels of halogenated olive oil indicate that halogenated fatty acids can be incorporated into the milk, be further absorbed into the neonate, and be metabolized in the process. This is in agreement with previous studies in which it was demonstrated that the label from iodinated fatty acids, fed to cows, appeared in the milk (16), and that the label from chlorinated fatty acids, fed to rats, appeared in both the milk and the neonate (17).

In the present study, the di-halogenated fatty acid degradation products are of particular interest in that in the milk lipids approximately 15-20% of the total halogenated acids are of shorter chain-length (C_{14} and C_{16}) than the original C_{18} dihalo acid, and that catabolism, presumably β-oxidation, stops at C_{14}. In the neonate liver lipids 55% of the di-halogenated acids are of shorter chain-length. This probably reflects further catabolism by the

neonate (18) but presumably could also result from selective absorption of the shorter chain fatty acid. Again catabolism appears to stop at C_{14} reflecting the apparent inability of the β-oxidation system to continue down the fatty acid chain. This can be contrasted with the catabolism in the adult rat of the 9,10-methylene octadecanoate which is apparently catabolized one stage further to the C_{12} 3,4-methylene acid (19). Both brominated and chlorinated lipids appear to be metabolized in a similar fashion.

REFERENCES

1. Takagi, Y,; Otake, T.; Kataoka, M.; Murata, Y.; Aburada, S.; Akasaka, S.; Hashimoto, K.; Uda, H., and Kitaura, T. (1976). Toxicol. Appld. Pharmacol. 38, 549-558.
2. Rickert, D.E.; Dent, J.G.; Cagen, S.Z.; McCormack, K.M.; Melrose, P., and Gibson, J.E. (1978). Environm. Persp. 23, 63-66.
3. Mes, J. and Davies, D.J. (1979). Bull. Environ, Contam. Toxicol. 21, 381-387.
4. Mes, J. and Davies, D.J. (1978). Chemosphere 9, 699-706.
5. Mes, J.; Davies, D.J., and Miles, W. (1978). Bull. Environ. Contam. Toxicol. 19, 564-570.
6. Jones, B.A.; Tinsley, I.J., and Lowry, R.R. (1983). Lipids 18, 319-326.
7. Jones, B.A.; Tinsley, I.J.; Wilson, G., and Lowry, R.R. (1983). Lipids 18, 327-334.
8. Lawrence, J.F.; Chadha, R.K.; Iverson, F.; McGuire, P., and Conacher, H.B.S. Lipids 19, 704-707.
9. Gough, B.M.; Whitehouse, M.E., and Greenwood, C.T. (1978). Critical Reviews in Food Science and Nutrition. 10, 91-113.
10. Sgoutas, D. and Kummerow, F.A. (1963). J. Am. Oil Chem. Soc. 40, 138-141.
11. Pihlaja, K. and Ketola, M. (1970). Suomen Kemistilehti, 43, 21-27.
12. Page, B.D. and Kennedy, B.P.C. (1975). J. Assoc. Off. Anal. Chem. 58, 1062-1068.
13. Bligh, E.G. and Dyer, W.J. (1959). Can. J. Biochem. Physiol. 37, 747-756.
14. Conacher, H.B.S.; Chadha, R.K., and Sahara-budhe, M.R. (1969). J. Am. Oil Chem. Soc. 46, 558-560.
15. Conacher, H.B.S., unpublished data.
16. Garton, G.A. (1963). Lipid Res. 4, 237-254.
17. Cunningham, H.M. and Lawrence, G.A. (1977). Fd. Cosmet. Toxicol. 15, 183-186.
18. Cunningham, H.M. and Lawrence, G.A. (1977). Fd. Cosmet. Toxicol. 15, 331-334.
19. Wood, R. and Reiser, R. (1965). J. Am. Oil Chem. Soc. 42, 315-320.

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Fatty Acid and Squalene Compositions of Mediterranean *Centrophorus* SPP Egg and Liver Oils in Relation to Age

D. PEYRONEL,[a] J. ARTAUD,[b,]* M.C. IATRIDES,[b] P. RANCUREL[c] and J.L. CHEVALIER,[a] [a]*Laboratoire de Chimie Appliquée et Génie Chimique, Université de Droit, d'Economie et des Sciences, Rue Henri Poincaré, 13397 Marseille Cedex 13, France,* [b]*Laboratoire des Organo-Phosphorés et Institut Universitaire de Technologie, Rue des Géraniums, 13337 Marseille Cedex 14, France,* [c]*Laboratoire de Zoologie Marine, Université de Droit, d'Economie et des Sciences, Rue Henri Poincaré, 13397 Marseille Cedex 13, France*

ABSTRACT

The liver oil content of seven Mediterranean *Centrophorus* spp. were studied together with the oil of one unfertilized egg and one yolk bag. The relative weight of the liver ranges from 15 to 29% of total body weight; 63 to 89% of the liver weight is oil. Squalene, the major component (49-89% in oil) of the unsaponifiable fraction, is directly quantified by GLC following TLC separation. The squalene level increases with the age of the animals. More than 50 fatty acid species were identified. Among them, the most abundant are: 16:0 (22-27%), 18:1ω9 (21-36%) and 22:6ω3 (2-18%). The level of ω3-fatty acids, essential in the fishes, shows a maximum in the egg (26%), decreases to a minimum in the young (5%) and stabilizes to an intermediate level in the adult (12%).
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INTRODUCTION

Centrophorus spp. is a small, cosmopolitan shark living at depths ranging from 150 to 1000 m. The liver, which is the main site of lipid accumulation in elasmobranchs (1), is one-fifth of the total body weight on the average. Squalene is the main constituent of the liver oil (2). Hydrogenation and purification of this oil lead to production of squalane, which is used as a fixative in the perfume industry and a skin lubricant in cosmetology. The various species are caught on the west African coasts (Senegal) and around Japan. Occasional captures of *Centrophorus spp.* are made on the Mediterranean French coast, but no commercial exploitation occurs.

An experimental deep sea fishery took place in 1982 on the Corsican coast to collect deep sea sharks: *Etmopterus pusillus, Hexanchus griseus, Galeus melanostomus* and *Centrophorus* spp. were taken. This unexploited *Centrophorus* liver oil may be of potential economic interest, so we have investigated its chemical characteristics, paying attention to its squalene content, and to its total fatty acid composition, and relate these to the physiological state of animals used in this study.

MATERIALS AND METHODS

The sharks were captured on long lines in the bay of Ajaccio (Corsica-France) during the year 1982, at depths ranging from 200 to 600 m. The juveniles and sub-adults were caught at shallower depths (150-180 m) than the mature animals (300-400 m).

Seven sharks were selected according to their physiological state and oil extracted from their livers. Moreover, one unfertilized egg (UFE) and one yolk bag (YB) were recovered from two females for fatty acid analysis. The biological data are presented in Table 1.

Systematics

Centrophorus spp. belongs to the large Squalidae family. The Squalidae are small, cosmopolitan sharks, either benthic or pelagic, and are found at all depths. Several species of *Centrophorus* live in the Mediterranean and Atlantic oceans, but their taxonomy is still not elucidated. Cadenat and Blache (3) have used sharks of identical sizes to discriminate between the species. The specific identification criteria are too labile and subtle during the growth period to be used safely. Thus we have considered that the Corsican species might belong to two "groups": *C. granulosus* (Schneider 1801) and *C. uyato* (Rafinesque 1820)-*C. machiquensis* (Maul 1955). Most of the sharks processed in this study belong to the group in which sexual maturity is reached at a relatively small size and we have assigned these to the *C. uyato-machiquensis* group.

*To whom correspondence should be addressed.

TABLE 1

Biological Data for Mediterranean *Centrophorus* spp.

Sample number[a]	UFE	YB	1	2	3	4	5	6	7
Sex			M	F	M	F	M	F	F
Length (cm)	12	10	34	55	60	65	82	101	100
Weight of animal (g)			223	785	1200	2320	4000	6400	6900
Weight of liver (g)			46	139	179	520	1150	1409	1673
Weight of egg (g)	310	210							

[a]UFE: unfertilized egg from female 6; YB: yolk bag of embryo 1; 1: male embryo from female 7; 2: mature female with poorly differentiated ovaries; 3: juvenile male with poorly differentiated testicles; 4: young female showing granular ovaries with oocyte diameters of more than 1 cm; 5: sexually mature male; 6: mature female with UFE; 7: female ending gestation.

TABLE 2

Analytical Data of *Centrophorus* spp. Liver Oil

Sample number[a]	1	2	3	4	5	6	7	Squalene
Saponification value	77	59	51	46	27	20	0	
Iodine value (Wijs)	247	273	295	306	345	333	308	360-380
Density, d_4^{25}	–	0.8625	0.8671	0.8531	0.8582	0.8593	0.8583	0.8584[b]
Refractive index, n_D^{25}	1.4843	1.4873	1.4880	1.4897	1.4914	1.4900	1.4903	1.4965[b]
Oil in liver (% wt)[c]	67	70	73	73	75	87	89	
Squalene in oil (% wt)[d]	48.8	58.7	66.3	71.8	88.6	79.7	82.3	

[a]See footnotes Table 1.

[b]Measured at 20 C.

[c]Results of hexane extraction. All other data refer to oil recovered without solvent.

[d]Determined by gas liquid chromatography.

Liver Oil Extraction

The sharks were deep-frozen at −30 C following their captures. The livers were removed in the laboratory, weighted and then shredded. The oil which exuded at ambient temperature without pressure (98% of total oil) was strained using paper and then stored under helium before being processed and analyzed. The oil weight percentages in the liver, reported in Table 2, take into account a complementary extraction using hexane at ambient temperature.

The iodine and saponification values, as well as refractive index and density, were determined according to AFNOR methods (4).

Squalene Quantification

Squalane was used as internal standard for quantification of squalene by gas liquid chromatography (GLC). Synthetic mixtures from 30 to 70% of squalene (Sigma, St. Louis, Missouri) and squalane (AEC, Commentry, France), purity over 98%, were used to set up a standard curve.

The pure squalane was added to the oil samples so the ratio of squalene on squalane weight approximated 1. Of this mixture, 25 μL at 10% (vol.) in hexane were deposited on silica gel plates Merck Si 60 F 254, 0.25 mm thickness and developed using hexane/ether (92:8, v/v) as the solvent system. Plates were sprayed with rhodamine B and bands were examined under 366 nm UV light. The squalene-squalane band (Rf = 0.9 − 1) was traced, scraped off and extracted with hexane (2-3 ml).

Mixtures were analyzed on an Intersmat IG 12 DFL gas chromatograph (France) equipped with a split injector and flame ionization detector. The silica capillary column used was 25 m long, 0.23 mm id and coated with OV 1701 (0.1 μm phase thickness). The column was operated at 230 C while the inlet and detector ovens were operated at 290 C. The inlet pressure of hydrogen used as the carrier gas was 0.8 bar (split 45 $ml \cdot min^{-1}$). Peak areas were integrated by a LTT ICAP 5 electronic integrator.

Analysis of Fatty Acid Composition by GLC

All the manipulations were carried out under helium. Following the saponification of oil with 2 M KOH/EtOH, the unsaponifiable

fraction was extracted using isopropyl ether (5). Fatty acid methyl esters were prepared by acid-catalyzed methylation using BF_3-CH_3OH (10%, Fluka, Buchs, Switzerland) according to AFNOR NFT 60-230 Norm (4). Commercial saturated and unsaturated, even and odd-numbered (Fluka), and polyunsaturated (Sigma, St. Louis, Missouri) fatty acid methyl esters were used as standards for the identification of *Centrophorus* liver oil fatty acid methyl esters, together with those of cod liver oil (6).

Analysis was performed on a Girdel 30 gas chromatograph (Girdel, France) equipped with a glass injector and a flame ionization detector. The glass capillary column was 40 m long, 0.30 mm id and coated with Carbowax 20 M, 0.15 μm d_f. Operating temperatures were 180 C for the column and 255 C for the inlet and detector ovens. Inlet pressure of hydrogen as carrier gas was 0.8 bar, split 40 $ml \cdot min^{-1}$; peak areas were integrated by LTT ICAP 5 electronic integrator.

Hydrogenation of Fatty Acid Methyl Esters

Platinum oxide (10% Pt, Fluka) deposited on charcoal (25 mg) was added to the mixture of fatty acid methyl esters (500 mg) in hexane (6 ml). Hydrogenation was achieved by stirring for 16 hr at ambient temperature under a slight positive pressure of hydrogen.

Gas Liquid Chromatography-Mass Spectrometry

Hydrogenated fatty acid methyl esters were analyzed on a Girdel-Ribermag R 10-10 B gas chromatograph mass spectrometer (Ribermag, France). The Girdel 30 chromatograph was fitted with a 25 m silica capillary column, 0.32 mm id coated with CP Sil 5 (Chrompack, France) 0.134 μm d_f. Operating conditions were: inlet 320 C, the column temperature linearly programmed from 140 to 300 C at 3 C min^{-1}, helium carrier gas 0.5 bar, ionizing voltage 70 eV, ion source 150 C. Data computation was performed on a Sidar system.

RESULTS

The relative weight of *Centrophorus* liver ranges from 14.9 to 28.8% of total animal weight. The analytical values summarized in Table 2 show variations with the age of animals: the oil percentage in the liver increases from 67 to 89% while the proportion of squalene in the oil increases from 49 to 89% (squalene is over 98% of unsaponifiable matter); the saponification value decreases from 77 to 16, while the iodine value increases from 247 to 345.

The squalene was identified by open-tubular GLC (OV 1701). The pristane often found in marine animals (7-10) was not detected in significant amounts.

The quantification of squalene was executed on raw oil, using squalane as internal standard. The squalene and the squalane were separated together from the other oil components (cholesterylester, diacylglycerylether, triglyceride) by TLC and analyzed by GLC (relative response factor $\simeq$ 1). Tests have shown the accuracy of this method (on synthetic mixtures of squalene-squalane or vegetable oil-squalene-squalane). Squalene quantitation in samples 1 to 7 shows a regular increase (49-89%) of this hydrocarbon as a function of age. A concomitant decrease of the saponifiable fraction is observed.

The fatty acid compositions of lipids from total UFE and YB samples, and of the liver oil from the seven shark samples, were determined as methyl esters by GLC (Carbowax 20 M). Chromatograms are characterized by more than 50 different acids identified by comparison with pure standards and with cod liver oil (6). Equivalent chain lengths (ECL) are derived from a second order equation relating the retention time log to the carbon number as described by Nelson (11). In our case of analysis for a large range of carbon atoms number (C12-C24), a linear relationship (12) gives a variance of $1.39 \cdot 10^{-3}$ while the variance given by a second order equation is $7.6 \cdot 10^{-4}$, which better fits the experimental curve. The ECL of saturated fatty acids (Table 3) were calculated to estimate their deviations from the theoretical values. The structural attributions lead to ECL values in good agreement with the previously published values on Carbowax 20 M (13-15) and allow assignment of structures to all observed peaks.

One unusual fatty acid was detected (ECL = 16.89). After hydrogenation of the esters, this component disappeared, and a peak of similar size (0.6-1%) was found at ECL = 16.26. It was identified by GC-MS (M^+ at m/e 284 with other ions at 241, 234, 185, 157, 129, 87, 74). The spectrum and fragmentation pattern were basically similar to that of methyl 7-methyl-hexadecanoate produced from 7-methyl-7-hexadecenoic acid found in liver oil of ocean sunfish *Mola mola* (16). Therefore, the esters were thought to contain a 7-methyl-7-hexadecenoic acid of ECL = 16.89 before hydrogenation. Still, it is possible that 7-methyl-hexadecanoic acid is also present and is hidden because its ECL is the same as that of 9-hexadecenoic acid. Complete results are given in Table 3. The same fatty acids were found in all samples and the important ones are = C16:0 (22.2-27.0%), C18:1ω9 (21.1-36.2%), C22:

TABLE 3

Fatty Acid Composition (Area %) of *Centrophorus* spp. Egg and Liver Oils

Sample number[a]	ECL[b]	UFE	YB	1	2	3	4	5	6	7	5Hydro[c]
Fatty acid											
12	11.96	–	–	–	–	–	–	–	–	–	tr
13	13.08	–	–	–	–	–	–	–	–	–	tr
14	13.99	1.3	1.1	0.9	2.1	2.1	2.9	2.3	1.5	1.5	2.2
iso 15	14.49	0.1	0.1	0.2	0.2	0.2	0.1	0.1	0.1	0.1	
anteiso 15	14.67	–	–	0.1	0.1	–	0.2	0.1	–	0.1	
15	14.98	0.5	0.5	0.4	0.9	0.5	0.9	0.7	0.5	0.5	0.6
iso 16	15.50	0.1	0.1	0.2	0.2	0.1	0.1	0.1	–	0.1	0.1
16	15.99	22.4	24.4	24.6	25.7	26.2	27.7	26.5	23.7	22.2	32.4
16:1ω9	16.20	0.9	1.3	0.7	0.5	0.6	0.5	0.8	1.2	2.0	
16:1ω7	16.26	3.8	3.6	3.8	5.4	4.6	6.6	5.9	4.5	4.5	
16:1ω5	16.40	0.3	0.4	0.2	0.2	0.4	0.3	0.3	0.2	0.3	
iso 17	16.53	0.2	0.2	0.3	0.3	0.3	0.1	0.4	0.2	0.3	0.2
anteiso 17	16.68	0.1	tr	0.3	0.2	0.1	0.1	0.1	–	0.1	0.2
7-M 7-H[d]	16.89	0.5	0.5	0.8	1.2	1.2	1.4	0.6	0.5	1.1	1.0
phytanic	16.96	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr
17	17.00	0.6	0.7	0.8	0.8	0.7	0.6	0.5	0.4	0.6	1.4
17:1ω8	17.23	0.7	0.7	0.8	1.1	0.9	1.0	0.8	0.8	0.8	
iso 18	17.50	0.1	0.2	0.4	0.4	0.3	0.2	0.1	0.1	0.2	0.2
18	17.97	2.3	2.9	3.4	3.6	3.5	3.2	4.0	2.4	2.9	37.8
18:1ω11	18.13	0.3	0.3	0.2	0.1	0.1	0.1	0.1	0.1	0.1	
18:1ω9	18.22	21.0	22.6	26.5	32.0	36.2	28.5	28.4	32.9	28.2	
18:1ω7	18.29	3.5	3.8	4.5	4.0	3.8	3.4	3.6	4.8	4.5	
18:1ω5	18.40	0.2	0.2	0.2	0.2	0.1	0.2	0.1	0.2	0.2	
18:2ω6	18.65	2.8	1.3	1.3	1.0	0.9	1.8	2.8	1.1	1.1	
18:3ω6	18.92	–	–	0.1	0.2	0.3	0.4	0.2	0.1	0.1	
19	19.01	0.1	0.2	0.1	0.2	0.1	0.2	0.1	0.1	0.2	0.6
19:1ω8 ?	19.22	0.1	0.2	0.2	0.2	0.1	0.1	0.1	0.2	0.2	
18:3ω3	19.25	0.6	0.5	0.4	0.3	0.2	0.5	0.6	0.3	0.5	
18:4ω3	19.54	0.1	–	0.1	0.2	0.2	0.4	0.4	0.1	0.1	
20	20.01	0.2	0.2	0.2	0.2	0.2	0.2	0.3	0.2	0.4	8.1
20:1ω11	20.14	0.6	0.6	0.6	1.4	1.1	0.6	0.7	1.1	1.2	
20:1ω9	20.19	2.7	3.0	3.2	4.2	4.2	3.1	2.9	4.5	5.6	
20:1ω7	20.26	0.2	0.2	0.3	0.4	0.3	0.3	0.3	0.4	0.4	
20:1ω5	20.44	0.1	tr	0.1	0.1	0.1	0.1	–	0.2	0.1	
20:2ω6	20.62	0.6	0.6	0.6	0.5	0.4	0.3	0.4	0.4	0.6	
20:3ω6	20.86	0.2	0.3	0.2	0.1	0.1	0.1	0.1	0.2	0.2	
21	21.01	tr	tr	tr	tr	tr	tr	tr	tr	tr	0.6
20:4ω6	21.06	3.1	3.8	2.8	1.1	0.7	1.1	1.1	0.5	0.5	
20:3ω3	21.24	0.1	0.2	0.3	0.1	0.1	0.1	0.1	0.3	0.3	
20:4ω3	21.48	0.2	0.4	0.3	0.3	0.2	0.2	0.3	0.4	0.5	
20:5ω3	21.68	4.0	3.0	2.1	1.5	1.1	2.0	1.8	0.9	1.2	
22	22.01	0.1	0.1	0.2	0.1	0.2	0.2	0.1	0.2	0.3	12.0
22:1ω11+1ω13	22.10	0.6	0.5	0.5	2.2	2.0	1.2	1.1	1.1	1.3	
22:1ω9	22.16	0.6	0.6	0.7	0.9	1.1	0.6	0.7	1.3	1.4	
22:1ω7	22.26	0.2	0.1	0.1	0.2	0.1	0.1	–	0.1	0.2	
23	23.00	tr	tr	tr	tr	tr	tr	tr	tr	tr	0.4
22:4ω6	23.04	0.7	1.0	0.9	0.3	0.2	0.3	0.3	0.7	0.8	
22:5ω6	23.30	0.9	0.9	0.7	0.4	0.3	0.5	0.4	0.6	0.5	
22:5ω3	23.66	3.4	3.0	2.7	0.7	0.4	0.5	0.8	2.3	2.6	
22:6ω3	23.92	18.0	15.1	11.3	2.7	2.1	5.6	7.3	7.1	7.9	
24	23.99	0.1	–	0.1	0.2	0.1	0.1	0.1	–	0.1	2.2
24:1ω9	24.15	0.8	0.6	0.6	1.1	1.4	1.2	1.5	1.5	1.4	
26	25.98	–	–	–	–	–	–	–	–	–	tr

[a]See footnotes Table 1.

[b]Equivalent chain length of fatty acid methyl esters on Carbowax 20M calculated by a second order equation giving relation between log d_R and carbon number.

[c]Hydrogenated esters of sample 5.

[d]7-methyl-7-hexadecenoic acid.

6ω3 (2.1-18.0%). Good agreement was found between fatty acid percentages before and after hydrogenation for sample 5. All chromatograms show a peak of residual squalene (ECL = 24.18), despite fatty acid-unsaponifiable fractionation, giving by hydrogenation a

peak of squalane (ECL = 19.74).

DISCUSSION

The relative weight of liver in Mediterranean *Centrophorus* spp. (14.9-28.8%), Japanese *Centrophorus* spp. (27.1%) (8) and Spanish *C. granulosus* (23%) (17) are of the same order of magnitude. The literature gives large variations in liver oil percentages for different species: *Centrophorus* spp., 88% (8); *C. granulosus,* 60-75% (17), 79-88% (18); *C. lusitanicus,* 86% (18); *C. jonsonii,* 34.8% (19), and *C. squamosus,* 35.5% (19).

An increase in percentage of unsaponifiable matter is correlated with aging of the animals. All the chemical characteristics of the oil become increasingly closer to those of squalene, which reaches a maximum value of 89% in the liver oil of an adult male (sample 5). A close value is given for *C. uyato* (90%) (20), although smaller values are reported for *Centrophorus* spp. and *C. granulosus* (8,17). It thus appears that the oil composition is a function of the animal's age. In this connection, the importance of precise biological data is stressed. The frequent lack of this data in previously published papers makes any comparison with our results difficult.

The occurrence of important quantities of squalene in liver oil is known for the deep sea sharks *Centrophorus, Centroscyllium, Etmopterus* and *Deania.* These oils have low densities ($d_4^{15} < 0.9$) by comparison to more common marine oils with high levels of triglycerides ($d_4^{15} > 0.9$), this value being used by Tsujimoto to classify shark oils (2). The most frequently advocated hypothesis to explain the function of liver oil in deep sea sharks is that it confers an almost neutral buoyancy (21). However, this hypothesis does not account for the significant amount of squalene in sharks living alternatively at the surface or at greater depths, e.g. *Cetorhinus maximus* (basking shark), or the absence of squalene in sharks sharing the same habitat with *Centrophorus,* like *Hexanchus griseus* (22). Other fats, such as diacylglycerylethers (23) and triglycerides (24), have been shown to play a role in buoyancy control.

To explain squalene accumulation in the liver, Kayama et al. (25) proposed that cholesterol biosynthesis follows the usual pathway but with the activation of hydrocarbon formation in lieu of completion of the sterol cyclization. Another hypothesis is the inhibition of cholesterol biosynthesis either by blocking of the 2,3 epoxysqualene formation resulting from a lack of monooxygenase (26), or by the inhibition of the 2,3 oxydosqualene cyclase. The latter process has been clearly demonstrated in plant tissue cultures (27,28) using various inhibitors (29-33) which lead to the accumulation of oxydosqualene. However, no evidence for the accumulation of squalene oxide in *Centrophorus* livers has been found.

Fatty acid composition of egg (UFE) oil was in good agreement with previously published results (34-36). However, a larger number of fatty acids were characterized and quantitated in this study. The fatty acid composition of liver oil was different from the one given by Kayama et al. (8), but comparisons are difficult due to the lack of biological data. The difference may be linked to the absence of species determination of sharks belonging to the *Centrophorus* genus, or to the occurrence of different habitats in the sampling localities. The percentages of saturated and unsaturated fatty acids of the same ω-position are reported for all samples in Table 4. Variations in ω-3 (linolenic acid family), essential for most fish (37), are important. The unfertilized egg which is the site of nutrient reserve for embryo development shows the highest level (26%) of ω-3 acids. Following the fertilization, this level decreases (22%) as a part of these fatty acids is transferred to the embryo for its growth and part is stocked in the embryo liver (17%), which has the highest level found in any shark liver. For the self-sustaining animals, the ω-3 level is minimal for the young (5%) owing to the growth process, while this level increases

TABLE 4

Sum (Area %) of Same ω-position Fatty Acid Classes in Egg and Liver Oils

Sample number[a]	UFE	YB	1	2	3	4	5	6	7
Total fatty acids (%)									
ω9	26.1	28.1	31.7	38.8	43.5	34.0	34.3	41.4	38.6
ω7	7.7	7.7	8.7	10.0	8.8	10.4	9.8	9.8	9.6
ω6	8.3	7.9	6.6	3.6	2.9	4.5	5.3	3.6	3.8
ω3	26.4	22.2	17.2	5.9	4.3	9.3	11.3	11.4	13.1
saturated	28.3	30.7	32.2	35.2	34.5	36.6	35.5	29.5	29.6

[a]See footnotes Table 1.

and stabilizes around 12% in adults. The curve showing the age and ω-6 acid relationship is similar, although smoother. The ω-6 acids, essential for man, improve the growth of some fish (38), but are usually found in small amounts in marine fish (39). The level of monoethylenic ω-7 fatty acids is almost constant for all the samples. The ω-9 and saturated fatty acid levels show a relative maximum correlated with the minimum level of ω-3 acids in the young animals.

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REFERENCES

1. Craik, J.C.A. (1978) J. Mar. Biol. Ass. UK 58, 913-921.
2. Tsujimoto, M. (1920) J. Ind. Eng. Chem. 12, 63-73.
3. Cadenat, J., and Blache, J. (1981) Requins de Méditerranée et d'Atlantique, Faune Tropicale XXI ORSTOM, Paris, 53-63.
4. AFNOR (1981) Recueil de Normes Françaises des Corps Gras, Graines, Oléagineux, Produits Dérivés, Afnor, Paris.
5. Castang, J.; Olle, M.; Derbesy, M., and Estienne, J. (1976) Ann. Fals. Exp. Chim. 69, 57-85.
6. Ackman, R.G.; Sipos, J.C., and Jangaard, P.M. (1967) Lipids 2, 251-257.
7. Mallins, D.C., and Wekell, J.C. (1971) Prog. Chem. Fats Other Lipids 10, 339-363.
8. Kayama, M.; Tsuchiya, Y., and Nevenzel, J.C. (1969) Bull. Jap. Soc. Sci. Fish. 35, 653-664.
9. Ackman, R.G. (1971) Lipids 6, 520-522.
10. Ackman, R.G. (1971) Lipids 6, 863-866.
11. Nelson, G.J. (1974) Lipids 9, 254-263.
12. James, A.T. (1952) Biochem. J. 52, 242-247.
13. Flanzy, J.; Boudon, M.; Leger, C., and Pihet, J. (1976) J. Chromatogr. Sci. 14, 17-24.
14. Mordret, F.; Prevot, A.; Le Barbanchon, N., and Barbati, C. (1977) Rev. Fr. Corps Gras 24, 467-475.
15. Ramananarivo, R.; Artaud, J.; Estienne, J.; Peiffer, G., and Gaydou, E.M. (1981) JAOCS 58, 1038-1041.
16. Ackman, R.G.; Safe, L.; Hooper, S.N., and Paradis, M. (1973) Lipids 8, 21-24.
17. Ayerbe, F.R., and Romero, A.A. (1955) Grassas y Aceites 6, 141-143.
18. Doutre, M.P. (1960) Bulletin de l'IFAN 22, 1433-1440.
19. Gastaud, J.M. (1969) Rapp. Comm. Int. Mer Medit. 19, 895-896.
20. Heller, J.H.; Heller, M.S.; Springer, S., and Clark, E. (1957) Nature 1979, 919-920.
21. Corner, E.D.S.; Denton, E.J.; Forster, F.R.S., and Forster, G.R. (1969) Proc. Roy. Soc. B. 171, 415-429.
22. Monforte, F., and Fenech, G. (1952) Chimica Industria 34, 804-814.
23. Malins, D.C., and Barone, A. (1970) Science 167, 79-80.
24. Ackman, R.G., and Eaton, C.A. (1970) J. Fish. Res. Bd. Canada 27, 1669-1683.
25. Kayama, M.; Zafar, S.; Rizwi, W., and Asakawa, S. (1971) J. Fac. Fish. Anim. Husb. 10, 1-10.
26. Gastaud, J.M.; Debay, P., and Balny, C. (1976) Rapp. Comm. Int. Mer Medit. 23, 53.
27. Benveniste, P., and Many-Westropp, R.A. (1967) Tetrahedron Lett. 37, 3553-3556.
28. Ponsinet, G., and Ourisson, G. (1967) Phytochemistry 6, 1235-1243.
29. Reid, W.W. (1968) Phytochemistry 7, 451-452.
30. Eppenberger, U.; Hirth, L., and Ourisson, G. (1969) Eur. J. Biochem. 8, 180-183.
31. Corey, E.J., and Ortiz de Montellano, P.R. (1967) J. Am. Chem. Soc. 89, 3362-3363.
32. Heintz, R., and Benveniste, P. (1970) Phytochemistry 9, 1499-1503.
33. Douglas, T.J., and Paleg, L.G. (1974) Plant. Physiol. 54, 238-245.
34. Shimma, H., and Shimma, Y. (1968) Bull. Jap. Soc. Sci. Fish. 34, 1015-1021.
35. André, E., and Canal, H. (1929) Bull. Soc. Chim. 45, 511-525.
36. Higashi, H.; Kaneko, T., and Suggi, K. (1953) Bull. Jap. Soc. Sci. Fish. 19, 861-867.
37. Takeuchi, T., and Watanabe, T. (1977) Bull. Jap. Soc. Sci. Fish. 43, 893-898.
38. Leger, C., and Fremont, L. (1981) in Nutrition des Poissons (Colloque CNERNA) pp. 215-246, CNRS edn., Paris.
39. Watanabe, T.; Takashima, F., and Ogino, C. (1974) Bull. Jap. Soc. Sci. Fish. 40, 181-188.

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Occurrence of a 22:2 Nonmethylene Interrupted Dienoic Fatty Acid and Its Seasonal Distribution Among Lipids and Tissues of the Fresh Water Bivalve *Diplodon delodontus* From an Isolated Environment

C.E. IRAZÚ,* R.J. POLLERO[1] and R.R. BRENNER,[2] *Instituto de Investigaciones Bioquímicas de La Plata (INIBIOLP), CONICET-UNLP, Facultad de Ciencias Médicas, calles 60 y 120, (1900) La Plata, Argentina*

ABSTRACT

Relatively high levels of a non-methylene-interrupted dienoic fatty acid were detected in the fresh-water mollusc *Diplodon delodontus*. The (7,13) 22:2 NMID fatty acid was separated from total fatty acids by TLC, and its structure was determined by GLC and reductive ozonolysis. Its seasonal distribution was investigated in different tissues and lipids of the mollusc. High concentrations of this acid were found in polar lipids. The absence of the 22:2 NMID fatty acid in the lipids of plankton and sediment in the same habitat suggests that it may be biosynthesized by the mollusc. Possible synthesis and functions of the NMID fatty acids are discussed.
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INTRODUCTION

Several isomers of 20:2 and 22:2 NMID fatty acids have been reported in marine molluscs such as bivalves and gastropods (1-5). Recent work in our laboratory demonstrated that a 22:2 NMID acid is present in the fresh-water bivalve mollusc *Diplodon patagonicus* (6). A fatty acid with similar chromatographic characteristics also was detected in the related species *D. delodontus*, which lives under different conditions in a distant habitat. In the present experiment the structure of this fatty acid, its distribution in different organs during the annual cycle of the animal and its relation to the fatty acid composition of the diet were investigated. The main objects of the experiment were to obtain further information about the presence of this type of acid in molluscs and about its possible origin.

In our special case, we collected molluscs living in a very particular habitat. The samples came from an isolated eutrophic pond in which the cyanophyceae constituted practically the whole of the phytoplankton.

MATERIALS AND METHODS

Samples

Specimens of *Diplodon delodontus* were obtained from a fresh-water pond close to the river Río de la Plata, Buenos Aires province, Argentina. Eight adult animals 8 to 12 cm long were collected each season (December, March, July and October) and transported to the laboratory in vessels containing water from the pond. Digestive diverticula, feet, gills, mantle and gonads of both sexes were separated and processed independently to obtain total lipids. Sediment samples (30 g) were collected each season from the same area. Planktonic algae samples, mainly composed of cyanophyceae of the genus Oscillatoria which predominated during the whole year, were obtained in winter, and during a bloom at late summer, with a 40 μ net.

Lipid Extraction and Fatty Acid Analysis

Tissues, sediment and plankton were homogenized and total lipids were extracted with chloroform/methanol (2:1, v/v) by the procedure of Folch et al. (7). Polar and neutral lipids were fractionated by silicic acid absorption chromatography (8). Each fraction was saponified with 10% KOH in ethanol during 45 min at 80 C under N_2. The unsaponifiables were extracted with petroleum ether and discarded. Fatty acids were extracted with petroleum ether and esterified with 3N HCl in methanol.

Neutral and polar lipids were analyzed by TLC on Silica gel G using hexane:ether:acetic acid (80:20:1, v/v/v) and chloroform:methanol: acetic acid:water (65:25:4:4, v/v/v/v). Lipids were identified by comparison of the Rf with standards or by spraying the plates with specific

*To whom correspondence should be addressed.
[1] Member of the Carrera del Investigador Científico, Comisión de Investigaciones Científicas de la Pcia. de Buenos Aires, Argentina.
[2] Member of the Carrera del Investigador Científico, Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina.

reagents. The quantitative determination of the lipids was performed by densitometry after charring with sulphochromic acid solution (9).

Fatty acid methyl esters (FAME) were analyzed by gas-liquid chromatography (GLC) in a Hewlett-Packard 5840 apparatus equipped with a flame ionization detector. A column packed with 10% SP-2330 on Chromosorb WAW was used. The oven temperature was programmed at 3 C/min from 140 C to 220 C. The chromatographic peaks were identified as described earlier (10).

The identities of some fatty acids were investigated by mass spectrometry (MS) in tandem with GLC, with a Hewlett-Packard 5995 GC-MS using the following conditions: source temperature 215 C, ionization potential 70 ev and scan speed 380 amu/sec. Helium was used as the carrier gas at a flow rate of 25 ml/min.

Isolation and Structural Analysis of 22:2 NMID Fatty Acid

Total FAME from each tissue were separated according to their number of double bonds by TLC, on preparative plates (500 μ) of Silica gel H impregnated with 10% $AgNO_3$. Chromatograms were developed by running them twice with hexane-ethyl ether-acetic acid (94:4:2, v/v/v) (11). The plates were sprayed with 2% dichlorofluorescein solution, and the spots were visualized under UV-light. Sample spots of Rf 0.74; 0.45; 0.29 and 0.19 corresponding to FAME standards of 0, 1, 2 and 3 double bonds respectively were obtained.

The zones corresponding to acids with two double bonds were scraped from the plates and eluted with chloroform-methanol-water (50:50:1, v/v/v). To separate 22:2 fatty acids from other dienoic fatty acids with different chain lengths, the extracts of fatty acids with two double bonds were again fractionated by TLC-$AgNO_3$ developed three times with chloroform (12) with the corresponding standards. The spots were detected in the same way as described above. The zones containing C_{22} dienoic fatty acids (Rf = 0.54) were scraped from the plates and eluted. Aliquots of these extracts were used to check their purity by GLC. Other aliquots were hydrogenated (13) and again analyzed by GLC to confirm the carbon chain-lengths.

The remaining extracts were used to determine the positions of double bonds by means of reductive ozonolysis according to the method of Stein and Nicolaides (14). Ozone generated by a micro ozonizer (Supelco) was bubbled through the solutions in pentane during 1-2 min in a dry ice-acetone bath. Ozonides were reduced by adding triphenylphosphine. The ozonolysis products were analyzed by GLC as described above, with the exception that the temperature was programmed 10 C/min from 100 C (10 min) to 220 C. Similar procedures were performed to determine the 20:1 fatty acid structure.

RESULTS

Structures of 22:2 and 20:1 Acids

The total FAME were fractionated by $AgNO_3$ TLC with hexane:ethyl ether:acetic acid (94:4:2, v/v/v). The fraction that corresponded to dienoic acids was analyzed by gas-liquid chromatography. It was shown to be made up principally of C_{22} and C_{18} acids, with only small amounts of C_{20} acids. By means of a new $AgNO_3$ TLC developed three times with chloroform, it was possible to separate zones that were made up of 22, 20 and 18 carbon dienoic acids. The purity of each zone was checked by gas-liquid chromatography before and after hydrogenation.

Reductive ozonolysis of the 22:2 methyl ester fraction followed by gas-liquid chromatography gave as products only two main peaks, about 45% each, corresponding to a C_7 aldehyde-ester and C_9 aldehyde (Table 1). Therefore, the structure of the acid may be attributed to a non-methylene-interrupted C_{22} acid with double bonds in the C_{7-8} and C_{13-14} positions. Other, minor peaks detected were less than 6%.

A similar separation of the methyl esters of C_{20} acids by $AgNO_3$-TLC followed by gas-liquid chromatography revealed the presence of two isomers. However, the low proportion of this fraction did not allow further study of the structure by ozonolysis.

The structure of the 20:1 acid also was investigated for possible metabolic relations to the 22:2 NMID acid. The corresponding fraction was separated by $AgNO_3$-TLC and ozonized. The structure corresponded to (11)-20:1 (Table 1). No other isomer was detected.

Composition of Lipids

The analysis of total lipids in December samples demonstrated that they were mainly polar lipids in proportions higher than 70% in all tissues except in digestive diverticula, where they totalled up to 52%. The principal lipid components were phosphatidyl ethanolamine, phosphatidyl choline, esterified sterol, free sterol, triacylglycerol, free fatty acids and alcohols.

The fatty acid compositions of neutral and polar lipids of different tissues of *D. delodontus* at different seasons are presented in Tables 2A,

TABLE 1

Structural Analysis of 22:2 NMID and of 20:1 Fatty Acids

Molecular ion (a)	Chain length of isolated acid (b)	Number of double bonds (c)	Ozonolysis products (d)		Double bond position
			Aldehyde ester	Aldehyde	
350	C_{22}	2	C_7	C_9	C_7, C_{13}
324	C_{20}	1	C_{11}	C_9	C_{11}

Determinations were performed by: a) GLC-MS; b) Hydrogenation followed by GLC; c) GLC after TLC-$AgNO_3$ separation; d) Reductive ozonolysis and GLC of products.

B, C and D. Only major fatty acids and those acids that show variations are included. The composition of total fatty acids of gonadal tissues of *D. delodontus* at different sexual stages was reported in a previous paper (10). It does not differ qualitatively from those found in other tissues in the present work. Table 2 shows that in all tissues analyzed, the predominant fatty acids are 16:0, 16:1, 18:1 and (11)-20:1. However, there are differences among the different tissues and between phospholipids and neutral lipids. In phospholipids the most abundant acids are in general 16:0, (11)-20:1 and (5,8,11,14)-20:4. The proportion of polyunsaturated acids in phospholipids is higher than in neutral lipids, while the proportion of 16:1 and 18:1 in neutral lipids is higher than in phospholipids. The 22:2 NMID is in general more abundant in phospholipids and is present in all the tissues analyzed. The highest value found was 9.8% in phospholipids and the lowest 1.8% in neutral lipids. The lipid composition of all the tissues showed variations during the yearly cycle.

Lipid and fatty acid composition of plankton and sediment collected from around the specimens also were analyzed. Neutral lipids were more than 90% and 80% of plankton and sediment lipids, respectively.

Free fatty acids and triacylglycerols were the largest components of sediment, while triacylglycerols were especially prominent in the plankton.

The composition of sediment fatty acids (Table 3) was rich in 16:0, 16:1, 18:1 and 18:0. The more abundant fatty acids in plankton were (9,12,15)18:3; 16:0 and 16:1. In all the samples analyzed the NMID 22:2 fatty acid was absent.

DISCUSSION

In a previous report (6) the structure of the NMID 22:2 acid of *D. patagonicus* was studied by mass spectrometry. The spectra corresponded to a (7,13)-22:2 and/or to a (7,14)-22:2 acid. In the present work, a mass spectrum of similar characteristics with a molecular ion of mass 350 was obtained for the NMID 22:2 acid of *D. delodontus,* and the same GC retention time was obtained. The analysis of the products obtained by reductive ozonolysis indicated 7,13 double bond positions. Other isomers apparently would be absent, since no other products of ozonolysis different from the C_7 aldehyde-ester and C_9 aldehyde were found in detectable amounts.

It has been suggested (15) that non-methylene-interrupted dienoic-fatty acids accumulate primarily in filter feeders such as shell fish or herbivores. The distribution of these anomalous fatty acid components in higher species suggests that they reflect invertebrates in their diet and are biochemically inert. Johns et al. (3) found that levels of NMID fatty acids in marine gastropods show a correlation with the levels of NMID acids present in the algal diet.

The examination of the fatty acid composition of the pond algae showed the presence and predominance of (9,12,15)-18:3, 16:0 and 16:1 fatty acids, and the absence of NMID 22:2 acid or of any immediate possible C_{20} precursor. None of these acids were detected in the fatty acids of the pond sediment, which was made up of algal and plant detritus in any season. Since both plankton and sediment form part of the mollusc diet, it is difficult to accept that the NMID 22:2 acid has an exogenous origin.

In *D. patagonicus* (16) after 60 days of starvation the NMID 22:2 acid level in the animal did not change. Moreover, the relative proportions of NMID 22:2 acid did not change when *D. patagonicus* was transferred to a different habitat and fed a quite different diet, whereas other fatty acid compositions changed significantly.

The composition of triacylglycerols is generally considered to reflect dietary fatty acid

TABLE 2A

Seasonal Changes in Fatty Acid Composition of Polar and Neutral Lipids Isolated from Specific Tissues of *D. delodontus*

		A – December (late spring)									
		Foot		Gill		Male gonad		Female gonad		Hepatopancreas	
Fatty acids	rrt[a]	PL	NL	PL	NL	PL	NL	PL	NL	PL	NL
16:0	0.73	16.2	20.4	10.1	20.1	13.5	19.0	12.9	14.0	21.8	16.7
(9)-16:1	0.79	12.1	12.7	11.5	13.8	6.4	17.5	15.3	24.2	9.6	15.1
18:0	1.00	7.2	10.0	4.4	12.3	5.0	6.4	4.3	1.6	7.2	5.3
(9)-18:1	1.06	10.9	10.6	6.1	7.9	6.9	9.8	9.1	11.9	7.5	9.8
(9,12)-18:2	1.15	4.5	3.4	4.4	4.7	4.4	3.5	4.7	5.1	4.8	5.4
(9,12,15)-18:3	1.27	3.3	2.0	5.8	5.4	7.7	6.4	9.7	11.1	7.6	9.6
(11)-20:1	1.33	17.5	7.2	15.1	7.2	16.3	5.4	10.7	7.8	11.4	7.4
(5,8,11,14)-20:4	1.54	6.4	4.9	11.4	1.9	11.6	2.9	5.7	1.7	3.4	3.9
(7,13)-22:2 NMID	1.65	6.3	2.5	5.3	7.2	6.9	2.3	4.4	3.5	2.9	3.0
(5,8,11,14,17)-20:5	1.67	1.3	2.3	2.7	?	3.8	2.9	3.1	1.5	2.0	2.3
Other[b]		14.3	24.0	23.2	19.5	27.5	23.9	20.1	17.6	22.3	21.5

[a]Retention time relative to 18:0.

[b]They include: 15:0; r-20:0 ?; 17:0; 17:1 ?; 22:3 ?; (7,10,13,16)-22:4; (10,13,16,19)-22:4; (7,10,13,16,19)-22:5 and (4,7,10,13,16,19)-22:6. This identification was performed under the conditions indicated in Materials and Methods.

TABLE 2B

	B – March (later summer)											
	Foot		Gill		Male gonad		Female gonad		Hepatopancreas		Mantle	
Fatty acids	PL	NL	PL	NL	PL	NL	PL	NL	PL	NL	PL	NL
16:0	10.2	18.7	12.7	14.0	13.3	25.1	14.0	17.6	17.0	17.7	13.2	16.4
(9)-16:1	6.0	17.3	5.6	18.8	6.7	9.4	6.5	15.7	5.8	10.1	5.6	17.7
18:0	6.9	10.2	5.5	9.0	8.0	24.6	7.4	7.5	7.5	7.1	6.1	9.5
(9)-18:1	7.8	15.9	4.4	11.1	7.2	13.6	7.2	13.5	5.8	8.4	5.9	13.3
(9,12)-18:2	5.0	4.1	5.2	3.8	3.4	2.4	5.2	2.3	6.6	5.8	4.8	2.7
(9,12,15)-18:3	4.3	1.6	6.4	5.8	2.4	1.2	5.9	3.9	12.7	11.2	4.9	1.8
(11)-20:1	20.6	8.3	20.7	13.0	20.0	4.9	17.3	8.5	14.1	6.9	18.6	11.8
(5,8,11,14)-20:4	11.2	4.1	10.3	3.7	8.0	3.4	8.7	5.0	6.9	6.1	13.4	3.5
(7,13)-22:2 NMID	8.7	1.8	7.9	2.9	8.2	2.3	8.9	3.2	3.1	3.1	8.9	3.5
(5,8,11,14,17)-20:5		0.9						1.9	4.7	2.7		
Others[a]	19.3	17.1	21.3	17.9	22.8	13.1	18.9	20.9	15.8	20.9	18.6	19.8

[a]They include: 15:0; r-20:0 ?; 17:0; 17:1 ?; 22:3 ?; (7,10,13,16)-22:4; (10,13,16,19)-22:4; (7,10,13,16,19)-22:5 and (4,7,10,13,16,19)-22:6. The identification was performed under the conditions indicated in Materials and Methods.

TABLE 2C

Fatty acids	C – July (winter)											
	Foot		Gill		Male gonad		Female gonad		Hepatopancreas		Mantle	
	PL	NL	PL	NL	PL	NL	PL	NL	PL	NL	PL	NL
16:0	10.4	14.0	9.3	14.3	12.5	15.3	11.8	14.6	9.5	14.2	9.1	13.8
(9)-16:1	9.2	14.7	12.7	21.4	12.1	23.6	16.8	23.6	6.9	14.2	5.9	17.2
18:0	4.5	9.2	3.3	3.5	7.5	2.4	4.4	1.6	4.8	6.7	3.6	7.7
(9)-18:1	7.7	13.1	6.9	12.7	9.4	13.0	7.8	11.7	4.9	11.6	5.4	16.1
(9,12)-18:2	5.5	4.5	5.6	5.0	6.0	5.1	4.8	5.3	4.1	4.0	4.9	4.6
(9,12,15)-18:3	5.7	2.1	9.9	7.8	6.2	9.2	12.1	14.5	10.7	5.5	4.8	2.5
(11)-20:1	15.6	18.1	11.2	12.3	11.9	7.6	9.2	8.3	11.1	11.6	16.1	12.3
(5,8,11,14)-20:4	13.2	3.4	8.1	1.8	5.2	1.3	5.6	1.6	7.4	3.8	13.9	3.1
(7,13)-22:2 NMID	6.3	2.7	7.3	3.5	8.2	3.7	4.8	4.3	9.8	2.6	8.7	3.0
(5,8,11,14,17)-20:5	2.4	0.9	1.3	0.7			3.4		1.4	1.6	2.0	0.7
Others[a]	19.9	17.3	24.4	17.0	21.0	18.8	19.3	14.5	29.4	24.2	25.6	19.0

[a]They include: 15:0; r-20:0 ?; 17:0; 17:1 ?; 22:3 ?; (7,10,13,16)-22:4; (10,13,16,19)-22:4; (7,10,13,16,19)-22:5 and (4,7,10,13,16,19)-22:6.
The identification was performed under the conditions indicated in Materials and Methods.

TABLE 2D

Fatty acids	D – October (early spring)											
	Foot		Gill		Male gonad		Female gonad		Hepatopancreas		Mantle	
	PL	NL	PL	NL	PL	NL	PL	NL	PL	NL	PL	NL
16:0	11.5	14.9	9.5	13.0	9.9	13.8	8.8	13.1	11.5	14.8	10.4	13.0
(9)-16:1	8.0	11.3	5.7	12.1	6.7	14.3	10.2	19.5	6.9	10.4	6.8	13.7
18:0	3.9	10.8	3.2	11.3	3.8	6.9	4.4	4.2	5.2	7.0	3.4	10.1
(9)-18:1	9.1	14.5	5.7	9.8	7.5	11.8	6.8	13.0	7.0	12.6	6.5	12.4
(9,12)-18:2	4.8	7.7	4.5	4.9	4.4	4.1	4.3	5.1	4.0	5.9	4.8	5.0
(9,12,15)-18:3	6.3	2.5	8.3	3.9	7.9	6.7	7.9	10.1	8.5	7.8	7.5	2.9
(11)-20:1	11.8	10.6	10.8	10.3	11.5	8.6	11.7	8.1	9.2	5.4	10.0	11.7
(5,8,11,14)-20:4	13.1	5.4	14.3	5.6	12.2	3.7	10.6	2.9	8.2	4.7	15.0	3.6
(7,13)-22:2 NMID	4.5	2.6	4.6	3.3	5.3	3.3	5.3	3.3	3.7	2.2	4.3	4.1
(5,8,11,14,17)-20:5	5.5	0.9	7.2	1.0	6.6	3.7	5.8	1.7	9.5	3.9	6.5	1.0
Others[a]	21.5	18.8	26.2	24.8	24.2	23.1	24.2	19.0	26.3	25.3	24.8	22.5

[a]They include: 15:0; r-20:0 ?; 17:0; 17:1 ?; 22:3 ?; (7,10,13,16)-22:4; (10,13,16,19)-22:4; (7,10,13,16,19)-22:5 and (4,7,10,13,16,19)-22:6.
The identification was performed under the conditions indicated in Materials and Methods.
Data are expressed as weight % calculated from GLC chromatograms. Only the principal components are listed.
Abbreviations used: PL, polar lipids; NL, neutral lipids.

TABLE 3

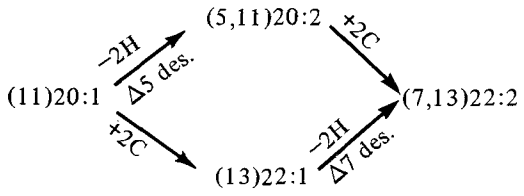
Fatty Acid Composition (%) of Sediment and Plankton from the Habitat of the Molluscs

Fatty acids	Sediment[a]		Plankton[b]
	Polar lipids	Neutral lipids	Total lipids
14:0	2.3	2.7	1.1
15:0+X	3.9	1.7	0.2
16:0	23.7	22.1	25.9
(9)-16:1	17.3	12.4	18.5
17 C	6.1	4.9	1.1
18:0	11.1	12.5	1.9
(9)-18:1	16.7	19.0	4.2
19:0 ?	0.7	0.5	–
18:2	4.8	7.6	8.8
(9,12,15)-18:3	1.7	3.3	30.8
20:1+18:4	1.8	2.4	0.9
20:2	0.9	1.2	–
21 C	1.3	0.4	–
20:4	3.1	2.7	0.6
(7,13)-22:2 NMID	–	–	–
Others	4.6	6.6	–

Fatty acids analyzed by GLC. Column packed with 10% SP-2330 on Chromosorb WAW.
[a]Results are the average of three determinations.
[b]Samples were composed mainly of Cyanophyceae of genus Oscillatoria and minor quantities of Chlorophyceae of genus Scenedesmus. Results are the average of two determinations.
X = Unknown.

composition. NMID 22:2 fatty acids are found in triacylglycerols, but they are present in phospholipids in a higher relative percentage. This would suggest a possible functional role of the NMID acid as well as a probable endogenous origin. The latter possibility also was suggested in a recent report by Klingensmith (4). The biosynthesis of non-methylene-interrupted acids (5,11)-20:2 and (5,11,14)-20:3 in rat liver microsomes has been shown by Ullman and Sprecher (17). On the other hand, the biosynthesis of (5,11,14,17)-20:4 and (5, 11,14)-20:3 from labeled α-linolenic and linoleic acids, respectively, was shown in transformed cells such as HTC cells (7288 C) (18,19).

A potential precursor of (7,13)-22:2 acid could be the (11)-20:1 acid which, after desaturation to (5,11)-20:2 by a Δ5 desaturase, would be elongated to (7,13)-22:2 acid. This pathway already has been suggested by Pearce and Stillway (12). Another possible route could be the Δ7 desaturation of a (13)-22:1 acid produced by elongation of (11)-20:1 acid.



No definite evidence exists yet for any of these pathways in molluscs. However, the Δ5 desaturase is an active enzyme in other animals, whereas the existence of a Δ7 desaturase is less documented. Moreover, the (11)-20:1 acid is very abundant in *D. delodontus* (Table 2) whereas (13)-22:1 acid was not detected in the animal. Therefore, the first route would be more probable than the second one, especially since Ullman and Sprecher (17), as well as Alaniz et al. (18), have shown that NMID polyunsaturated acids may be synthesized by a Δ5 desaturase from unsaturated acids of C_{20} possessing a double bond in the Δ11 carbon.

It has been suggested (15) that NMID 20:2 and 22:2 fatty acids would be biologically inert for higher species. It also has been claimed that the proximity of a double bond to the carboxyl group increases the stability of NMID fatty acid esters towards lipolytic hydrolysis. Therefore, the accumulation of NMID fatty acid in external membranes of molluscs would increase the protection against the attack of microbial lipases (1). It might explain the highest accumulation of NMID fatty acids in mantle and gills of *M. mercenaria* (4). In *D. delodontus* NMID 22:2 acid also is found in mantle and gills, but it is not possible to deduce from our data that the acid is preferentially incorporated in this tissue (Table 2). Probably more profitable speculation may be made from the data in Table 2 that show a small preferential incorpo-

ration of NMID in the phospholipids. Since phospholipids are obligatory components of membranes and the parameter order and structure of a lipid bilayer are in some aspects related to the hydrocarbon tail characteristics of the fatty acid, it is possible that a NMID double bond structure may have special effects on the membrane. We intend to study the effect of NMID fatty acids on the physicochemical properties of lipid bilayers.

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REFERENCES

1. Ackman, R.G., and Hooper, S.N. (1973) Comp. Biochem. Physiol. 46 B, 153-165.
2. Paradis, M., and Ackman, R.G. (1975) Lipids 10, 12-16.
3. Johns, R.B.; Nichols, P.D., and Perry, G.J. (1980) Comp. Biochem. Physiol. 65 B, 207-214.
4. Klingensmith, J.S. (1982) Lipids 17, 976-981.
5. Ackman, R.G. (1982) Proceedings of Second Int. Conference on Aquaculture Nutrition, Delaware, 1981.
6. Pollero, R.J.; Brenner, R.R., and Gros, E.G. (1981) Lipids 16, 109-113.
7. Folch, J.; Lees, M., and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497-509.
8. Wren, J.J. (1960) J. Chromatogr. 4, 173-195.
9. Blank, M.L.; Schmit, J.A., and Privett, O.S. (1964) J. Am. Oil Chem. Soc. 41, 371-376.
10. Pollero, R.J.; Irazú, C.E., and Brenner, R.R. (1983) Comp. Biochem. Physiol. Part B, 76, 927.
11. Dudler, P.A., and Anderson, R.E. (1975) Lipids 10, 113-115.
12. Pearce, R.E., and Stillway, L.W. (1977) Lipids 12, 544-549.
13. Farquhar, J.W.; Insull, W.; Rosen, P.; Stoffel, W., and Ahrens, R.H. (1959) Nutr. Rev. 17, 29-30.
14. Stein, A.R., and Nicolaides, N. (1962) J. Lipid Res. 3, 476-478.
15. Paradis, M., and Ackman, R.G. (1977) Lipids 12, 170-176.
16. Pollero, R.J., and Brenner, R.R. (1981) Lipids 16, 685-690.
17. Ullman, D., and Sprecher, H. (1971) Biochim. Biophys. Acta 248, 186-197.
18. Alaniz, M.J.T. de; Gómez Dumm, I.N.T. de, and Brenner, R.R. (1976) Mol. Cell. Biochemistry 12, 3-8.
19. Alaniz, M.J.T. de; Gómez Dumm, I.N.T. de, and Brenner, R.R. (1977) Advances in Exp. Med. and Biol., Function and Biosynthesis of Lipids, Vol. 83, pp. 617-624, Plenum Press.

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The Distribution of Serum High Density Lipoprotein Subfractions in Non-Human Primates[1]

CHARLES A. NELSON,*[a] WILLIAM E. GREER,[b] and MANFORD D. MORRIS,[a]
[a]Departments of Biochemistry and Pediatrics, University of Arkansas for Medical Sciences, Little Rock, Arkansas 72205 and [b]Gulf South Research Institute, New Iberia, LA 70560

ABSTRACT

The ultracentrifugal flotation patterns in 1.2 g/ml solvent and ultracentrifugal gradient distribution of high density lipoproteins (HDL) from the primates–human, apes and monkeys–were determined, with emphasis on the gorilla species of apes and rhesus monkeys. Diets for non-human primates were commercial chow, which is low in cholesterol. Molecular weights and protein, cholesterol, phospholipid and triglyceride compositions of various density fractions were determined on human, gorilla and rhesus HDL. The HDL_2/HDL_3 ratio was determined from the two peaks observed upon flotation in high salt in the analytical ultracentrifuge. The HDL_2 of all three species of apes–gorillas *(Gorilla gorilla)*, chimpanzees *(Pan troglodytes)* and orangutans *(Pongo pygmaeus)*–was always greater than HDL_3, while that of all six species of Old World monkeys–Rhesus *(Macaca mulatta)*, sooty mangabeys *(Cercocebus atys)*, cynomolgus *(Macaca fascicularis)*, stumptails, *(Macaca arctoides)* patas *(Erythrocebus patas)* and African greens *(Cercopithecus aethiops)*–was less. In addition, the HDL_3 concentration in five gorillas was about 15 mg/dl as cholesterol while the HDL_2 concentration was 92 mg/dl, much lower and higher, respectively, than humans. HDL_2 of gorillas was similar in density and molecular weight to that of humans. The distribution of densities in gorilla HDL was predominantly in HDL_2, while rhesus HDL usually, but not always, was unimodal, having a density distribution similar in heterogeneity to human HDL_3, but somewhat less dense (peaking at 1.109 vs 1.129 g/ml). The molecular weight of rhesus HDL was about the same as human HDL_3 in all three density subfractions and at the peak density. Likewise, the chemical compositions were similar for the subfractions 1.10-1.125 and >1.125 g/ml for rhesus HDL and human HDL_3. Consequently most but not all chow-fed rhesus HDL was very similar to human HDL_3, but lighter in density.
Lipids 19:656-663, 1984.

INTRODUCTION

Fewer detailed studies have been carried out on serum lipoproteins of apes than of monkeys. Apes are more similar to humans in many characteristics, but monkeys are more economical and are easier to adapt to experimental situations. It would be of interest to compare the groups–humans, apes and monkeys. Recently, HDL have assumed importance for their role in plasma cholesterol transport, but efforts to define their precise role have not met with success. In view of the heterogeneity of the high density lipoproteins, we wished to extend the available information to apes and compare the distribution and physical properties of HDL among humans, apes and monkeys. To this end, we have isolated and characterized, under similar conditions, the HDL and density gradient subfractions of gorillas, orangutans and chimpanzees, in addition to performing similar studies on three different species of monkeys: cynomolgus, rhesus and sooty mangabey.

[1] A preliminary report of this study was given at the American Society for Biological Chemists Meeting in New Orleans in April 1982.

*To whom correspondence should be addressed.

METHODS AND MATERIALS

All animals were fed Purina chow and housed at the Gulf South Research Institute at New Iberia, Louisiana, except for the cynomolgus which were at the University of Arkansas for Medical Sciences at Little Rock. Animals were not fed overnight. Blood was collected and allowed to clot, after which dithionitrobenzoic acid, thimerosal and EDTA were added to the serum. The serum was shipped immediately in ice and arrived iced within 24 hr. Total cholesterol (1), HDL cholesterol (2), triacylglycerol (3) and agarose electrophoresis (4) were carried out on each sample. Human low density lipoproteins (LDL) were samples described previously (5). All but one were male and of ages 47-61.

Purification–HDL was purified by one of two methods. The first was ultracentrifugation between KBr solvents of densities 1.063 and 1.25 g/ml, including a layer of 1.22 g/ml solvent which served to wash the floating HDL, particularly well in the swinging bucket rotor. The other method involved flotation at 1.22 g/ml through a layer of solvent, followed by 6% agarose column gel filtration which

separates according to molecular size (6). HDL was pure by agarose electrophoresis and analytical ultracentrifugation.

HDL_2 and HDL_3–HDL, upon flotation velocity in density 1.2 g/ml KBr at 48,000 rpm in a double sector cell in the Beckman Model E analytical ultracentrifuge, separated into two components, which were well defined. HDL_2 and HDL_3 were clearly visible as shown in the top photograph of Figure 1. The photographs were enlarged 10X and traced on graph paper. The peak tracings were redrawn to a linear baseline. The two components were estimated by completing tracing of the HDL peak or peaks symmetrically. All area under the HDL peak could be accounted for by two components. A figure illustrating the method has been given previously (7). The relative areas were corrected for the sector shape of the ultracentrifuge cell. No correction was made for the Johnston-Ogston effect since it is about 2-3% (8). There was no relative area change of HDL_2 and HDL_3 upon dilution. Remixing purified HDL_2 and HDL_3 yielded the correct ratios.

Flotation velocity rate measurements were carried out on 3-10 mg/ml solutions of HDL dialyzed vs 1.2 g/ml KBr solvent containing 0.01% Na azide plus 10^{-4}M EDTA at pH 7.5. The exact density was measured pycnometrically for each solvent after dialysis. A double sector cell was employed to obtain a baseline. The speed was 48,000 or 47,660 rpm at 25 C and a schlieren bar angle of 60 degrees.

Equilibrium molecular weights were carried out at 4-7 C (the exact temperature was measured) in solvents of NaBr containing 0.01% Na azide plus 10^{-4}M EDTA at pH 7.5 near 1.43 g/ml. (The exact density was measured with a Westphal balance at the temperature used.) Equilibrium was attained in 48 hr at speeds of 10,000-13,000 rpm using 0.15 ml of solvent dialyzed HDL, which had been diluted with solvent to a concentration of 0.3-0.5 mg/ml. Interference optics was used. The reciprocal of the density of the sample was taken as the partial specific volume. This method has been described previously for human HDL subfractions (9) and is similar to that used for LDL (10).

Density gradients–In preliminary experiments, density gradients were prepared from a number of salts. In the swinging bucket tubes, gradients have the highest resolution, but require very long equilibrium times. We found KBr to yield steep gradients, particularly at the bottom end. NaCl, on the other hand, could be made nearly linear and ranged over 0.06 g/ml, as from 1.07 to 1.13 g/ml. NaBr was intermediate. Consequently we selected NaCl or NaBr for the density gradients. Gradients were prepared by stepwise layering of 5 salt solutions of NaBr or NaCl with different densities containing 0.01% azide and 10^{-4}M EDTA in, for

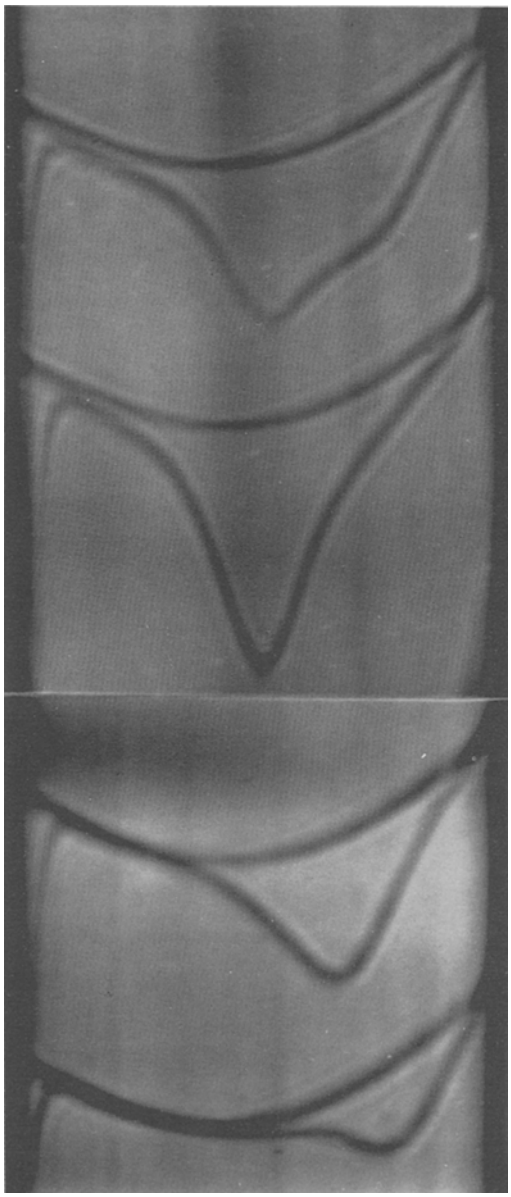

FIG. 1. From top to bottom; A. gorilla #3 HDL containing 57% HDL_2; B. gorilla #6 HDL containing 91% HDL_2; C. rhesus #7818 HDL; D. human HDL containing 89% HDL_3. All were in density 1.2 ± 0.005 g/ml KBr solvent at 48000 rpm, 25 C. Flotation is from right to left. The photographs were taken 80 min after reaching speed. Note the intermediate flotation rate of rhesus HDL. The flotation rates (S_f, 1.2) of the four major peaks were (in the same order): 6.6, 6.5, 4.6, and 2.8.

example, 1.07, 1.08, 1.10, 1.11 and 1.13 g/ml densities. Dialyzed HDL was added through a pipette as the middle fraction. Centrifugation for 72 hr at 15 C was in the Beckman SW40 rotor at 36,000 rpm in 6 tubes of 13 ml each. No brake was used at the end of each run. A hole was punched in the bottom of each tube and 15 fractions were collected. The density of each fraction was measured at room temperature by pycnometry. Absorbancies at 280 nm were determined to locate the protein portion of the lipoprotein. Cholesterol measurements paralleled the protein but peaked in density fractions 0.002-0.005 g/ml less.

Chemical analyses were–Protein by the Hartree method (11) using bovine serum albumin as standard, and phospholipid from phosphate (12), multiplying by 25 to correct for the added weight of lipid. The cholesterol ester content was assumed to be 70% and the cholesterol content multiplied by 1.5 to correct for the added ester weight. All errors are standard deviations.

RESULTS

Lipoprotein distribution–The gorillas had the highest total plasma cholesterol and HDL cholesterol of any of the groups of animals. About 40% of the total plasma cholesterol was found in HDL. The mean total cholesterol value of the orangutan and chimpanzee was about 200 mg/dl, and 30-35% of this was found in HDL. For comparison, we have taken values from the data of Srinivasan et al. (13) on chimpanzees. Their mean total cholesterol values are somewhat lower than ours, but the % distribution of cholesterol in HDL is identical to that found by us. The most variable total cholesterol was found in the three groups of sooty mangabeys where the free ranging group from Yerkes had the highest total cholesterol when compared to the caged animals. The % of HDL cholesterol was 41-43% in each group of sooty mangabeys (Table 1).

The rhesus monkeys transported about 47% of total cholesterol as HDL cholesterol. Srinivasan et al. (13) found, in a small group of rhesus monkeys, greater than 50% of total cholesterol as HDL. The cynomolgus had both the lowest total plasma cholesterol (115 mg/dl) and the highest % HDL cholesterol (about 50%) of any group of apes or monkeys. VLDL is not shown separately, but was very low (2-5 mg/dl) in all animals. Triglycerides were likewise low.

The apes had a lower proportion of their lipoproteins in HDL. However, the absolute amount of HDL was no less than that of the monkeys. Gorillas had the highest mean HDL concentrations. The lowest HDL concentrations were in chimpanzees, cynomolgus and some of the sooty mangabey groups.

HDL_2 and HDL_3 distribution–HDL_2 and HDL_3 separated sufficiently upon flotation velocity in density 1.2 g/ml KBr solvent in the analytical ultracentrifuge to be estimated. Shown at the top of Figure 1 are the HDL from the two gorillas having the least HDL_2 (1A) and the most HDL_2 (1B). Human HDL_3 is shown at the bottom of Figure 1 (1D). It moved slightly slower than gorilla HDL_3 (right hand peak in the top sample). This can be seen upon careful comparison of the two peaks. Chow-fed rhesus HDL usually was anomolous, having a single

TABLE 1

Distribution of Cholesterol in Chow-Fed Primate Lipoproteins

Species	N[a] (number of determination)	VLDL + LDL mg/dl Total	HDL	%HDL
Apes				
Gorilla	5 (13)	272 ± 85	159 ± 49	110 ± 44	40.4 ± 4.4
Orangutan	2 (3)	190 ± 54	121 ± 74	69 ± 21	35.0 ± 14.0
Chimpanzee (ours)	43	203 ± 48	140 ± 31	60 ± 16	30.0 ± 5.9
Srinivasan et al. (13)	13	167 ± 27	117 ± 24	50 ± 19	30.0 ± 9.6
Monkeys				
Sooty Mangabey				
Juvenile	6 (30)	149 ± 31	88 ± 26	60 ± 12	40.8 ± 6.0
Adult	11 (53)	135 ± 30	81 ± 24	55 ± 13	42.6 ± 9.9
Yerkes (7)	20	187 ± 40	106 ± 28	81 ± 9	43.5 ± 6.6
Rhesus (ours)	142	155 ± 31	81 ± 21	73 ± 16	47.0 ± 6.0
Srinivasan et al. (13)	16	141 ± 29	63 ± 20	78 ± 22	57.8 ± 13.9
Cynomolgus (ours)	14 (44)	115 ± 25	57 ± 17	58 ± 14	51.6 ± 11.5
Rudel and Lofland (14)	63	125	71	54	43.0

[a]N = no. of animals. In parentheses is the number of total measurements.

TABLE 2

Distribution of HDL Subfractions in Chow-Fed Non-Human Primates

Species	N (determinations)	HDL Cholesterol mg/dl	HDL_2 Weight per cent	HDL_3 Weight per cent
Apes				
Gorilla	5 (13)	110 ± 44	77 ± 18	23 ± 18
Orangutan	2 (3)	69 ± 21	72 ± 16	28 ± 16
Chimpanzee	5	63 ± 11	75 ± 16	25 ± 16
Monkeys				
Sooty mangabey	14 (20)	57 ± 13	33 ± 25	67 ± 24
Rhesus	19[a]	73 ± 14	0	100

The distribution of HDL into HCL_2 and HDL_3 was determined from the two peaks observed upon flotation velocity in the analytical ultracentrifuge in density 1.2 g/ml KBr solvent. They were recalculated to cholesterol content to correspond to the total HDL column.

All ape species were composed of 2 males with the remainder females. Sooty mangabey were 3 males, 11 females, while the rhesus included 10 males. Gorilla and chimpanzee males had about the same % HDL_2, while the three sooty mangabey males had 61 ± 8% HDL_2 and 69 mg/dl mean HDL cholesterol.

[a]Not included were two rhesus which had 2 HDL components and 3 rhesus which had 1 HDL component which was intermediate in flotation between the usual two peaks.

peak floating intermediate in speed between human HDL_2 and HDL_3 (Fig. 1C). Among any group of rhesus were a few with a clearly discernable HDL_2 and HDL_3 and a few with a faster single peak than usual, although not an HDL_2.

The distribution of HDL between HDL_2 and HDL_3 among apes is shown in Table 2 along with the sooty mangabey and rhesus. In every individual ape serum, HDL_2 was greater in amount than HDL_3 (Fig. 2). This is in contrast to all monkey groups such as the mangabeys and rhesus where the mean HDL_2 levels were less than the HDL_3 levels. We also studied two stumptails, three patas and two African green monkeys. Their HDL_3 predominated also, being 94 ± 6%, 90 ± 2%, and 95 ± 5%, respectively. We found in cynomolgus HDL, low S_f HDL (like HDL_3) also predominated (15).

Density gradient distribution–To clarify the comparative structure of primate HDL, equilibrium density gradient ultracentrifugation was carried out on numerous samples, three of which are shown in Figure 3. Gorilla #3 HDL (57% HDL_2) peaked at a very low density, 1.080 g/ml. Rhesus #7911 HDL peaked near that of human HDL_3, 1.110 vs 1.115 g/ml. Gorilla #6 HDL, which contained 91% HDL_2, contained little material in the region above 1.10 g/ml. Rhesus HDL from animals with no discernible HDL_2 and HDL_3 flotation velocity peaks contained considerable HDL below 1.10 g/ml density. Human HDL_3 spread over a similar density range (1.10-1.15 g/ml) as found by Cheung and Albers (16).

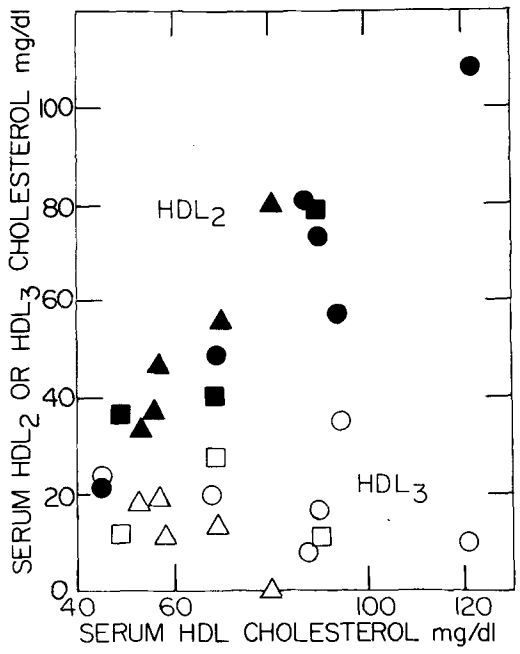

FIG. 2. The amount of HDL_2 and HDL_3 as a function of total serum HDL cholesterol concentration in mg/dl in apes. The closed symbols are HDL_2; the open symbols are HDL_3. Circles are gorillas, triangles are chimpanzees and squares are orangutans.

The density distribution of cholesterol found in HDL for primates is shown in Table 3. The contrast of gorilla HDL with rhesus HDL and human HDL_3 is evident, the former having 73-89% HDL cholesterol in the <1.10 g/ml

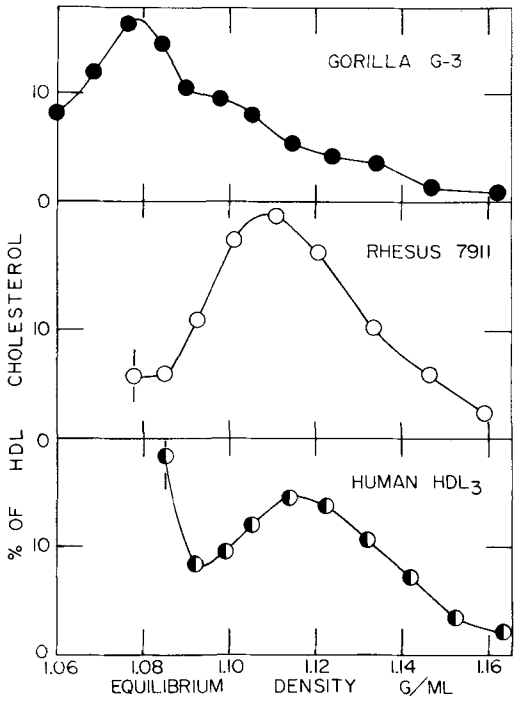

FIG. 3. Equilibrium density gradient patterns of gorilla #3 (top), rhesus #7911 (middle), and a human (bottom) having very little HDL_2 (3% by flotation velocity). The gorilla chosen was that having the least amount of HDL_2 (57% by weight).

density range, while rhesus and human HDL_3 had about 30% in that range. Human HDL_3 was broader in density distribution than rhesus HDL, and contained more material of density >1.125 g/ml.

Comparative peak fraction densities, flotation rates of the major component and flotation equilibrium molecular weights of this peak fraction are given in Table 4. While the gorilla peak fraction was clearly an HDL_2, being of 400,000 molecular weight, rhesus HDL and human HDL_3 were nearly the same at 209-200,000 molecular weight. Rhesus HDL floated faster in density 1.2 g/ml solvent because it had a lighter density, as seen in both Tables 3 and 4.

TABLE 3

Density Distribution of HDL

	N	Density ranges in g/ml <1.10	1.10 -1.125	>1.125
		% as cholesterol		
Gorilla[a] #6		89	8	3
HDL #3		73	19	8
Rhesus	7	30	53	17
HDL		±13	±14	±7
Human[b]	15	29	37	34
HDL_3		±9.5	±6	±12

[a]The gorillas used were one having little apparent HDL_3 (9%) and one having the highest amount of HDL_3 (43%).

[b]Individuals chosen who had 87 ± 6% HDL_3 (the denser of the two HDL peaks observed upon flotation velocity).

The gorillas and mangabeys had higher HDL_2 flotation rates than orangutans and chimpanzees, while HDL_3 S_f were similar. Orangutan HDL_3 S_f values were too few and at too low an HDL_3 concentration to consider. The peak fraction was determined from protein measurement (Table 4), which was at a higher density (0.002-0.006 g/ml) than cholesterol measurements revealed (Table 3).

Molecular weights--Human HDL_3 is heterogeneous (17). To determine whether rhesus HDL contained only HDL_3-like molecules, flotation equilibrium molecular weight determinations were carried out on various density fractions (Table 5). Between 1.11 and 1.14 g/ml, molecular weights ranged only from 216 to 189,000 for either rhesus HDL or human HDL_3. In density 1.085-1.090 g/ml, HDL near 290,000 was found in both.

TABLE 4

Densities and Sizes of the Primate HDL Peak Density Gradient Subfraction

	N	Density g/ml	Flotation rate S_f, 1.2	Molecular weight
Gorilla HDL	5	1.077 ±0.001	7.3± 0.5	400,000
Rhesus HDL	10	1.109 ±0.005	4.6± 1.0	209,000± 9,000
Human HDL[a]	20	1.129 ±0.012	3.5± 0.4	200,000± 15,000

N for molecular weights were 1,5 and 10 respectively. All mean values and standard deviations are of the peak fraction in each density gradient.

[a]Individuals chosen who had little HDL in the lower density of the two HDL peaks observed upon flotation velocity.

TABLE 5

Molecular Weight of HDL Density Gradient Subfractions

	N	Density fraction of HDL in g/ml		
		1.085 -1.090	1.109 -1.114	1.133 -1.140
		Molecular weight X 10^{-3}		
Rhesus HDL	5	291 ± 26	209 ± 9	189 ± 12
Human HDL[a] ($87 \pm 6\%$ HDL_3)	8	284 ± 28	216 ± 17	195 ± 16

[a]Individuals chosen who had $87 \pm 6\%$ HDL_3.

TABLE 6

Comparative Chemical Composition of Primate Density Gradient HDL Subfractions

Density subfraction	N	Protein	Cholesterol	Phospholipids	Triacylglycerol
			Weight percentage		
Gorilla HDL_2	2	37 ± 2	29 ± 0.5	29.6 ± 0.6	4 ± 2
Rhesus HDL 1.1-1.125 g/ml	12	48.2 ± 2.6	16.0 ± 2.3	30.3 ± 2.9	5.3 ± 2.7
Rhesus HDL > 1.125 g/ml	6	59.7 ± 2.3	14.9 ± 1.0	24.1 ± 1.9	1.3 ± 1.5
Human HDL_3 1.1-1.125 g/ml	6	49.2 ± 6.1	17.3 ± 1.2	27.4 ± 3.8	5.0 ± 2.0
Human HDL_3 > 1.125 g/ml	6	56.5 ± 5.0	15.7 ± 1.4	23.5 ± 2.2	4.3 ± 2.0
Rhesus HDL 1.063-1.125 g/ml Scanu et al. (18)		45.0 ± 5	20.4 ± 4	24.0 ± 5	3.2 ± 0.5
Rhesus HDL 1.125-1.21 g/ml Scanu et al. (18)		53.0 ± 5	16.7 ± 3	26.5 ± 5	2.8 ± 0.5
Rhesus HDL 1.063-1.125 g/ml Portman et al. (19)	11	51.1 ± 2.0	21.1 ± 1.6	25.9 ± 1.4	1.5 ± 0.3
Rhesus HDL 1.125-1.21 g/ml Portman et al. (19)	11	55.3 ± 5	20.5 ± 1.2	22.8 ± 0.5	1.4 ± 0.2

Chemical compositions were carried out on fractions in density ranges 1.063-1.125 and >1.125 g/ml (Table 6), in order to assess their heterogeneity and to compare them to previous studies of rhesus HDL (18,19), and to human HDL_{2b} and HDL_3 (20). While pure gorilla HDL_2 clearly was similar to human HDL_2 in composition, rhesus HDL and human HDL_3 were similar to each other.

DISCUSSION

HDL_2/HDL_3 ratios of apes and monkeys– Our major finding was that the HDL of apes, gorillas, chimpanzees and orangutans contained more HDL_2 than HDL_3, while that of Old World monkeys, rhesus, sooty mangabeys, cynomolgus, stumptails, patas and African greens, contained less HDL_2 than HDL_3 on a low cholesterol, low fat diet. This separation and estimation of HDL_2 and HDL_3 was based solely on the natural separation into two symmetrical, floating peaks in density 1.2 g/ml solvent in the analytical ultracentrifuge. While we have described the uncertainties in assigning rhesus HDL and HDL_2 or HDL_3 designation, this does not obscure the marked lower density, lipid-rich and higher molecular weight of most of ape HDL. Ape HDL is unique in containing less HDL_3 than even human HDL in spite of the fact that the total concentration of HDL cholesterol was much higher. Since HDL_2/HDL_3 ratios are regulated by hepatic lipase and plasma lipoprotein lipase (21), possibly either hepatic lipase is low, or lipoprotein lipase is high, or both, in apes. Hepatic lipase hydrolyzes phospholipid from HDL_2 preferentially (22,23), while lipoprotein lipase stimulates the formation of HDL_2 from surface constituents of VLDL and chylomicrons (24,25).

HDL concentrations of ape and monkey sera– Both apes and monkeys had higher serum HDL concentrations than humans. Some sooty mangabeys and chimpanzees had serum HDL concentrations similar to humans having high concentrations (50-60 mg/dl as cholesterol). Serum low density lipoprotein (LDL) concentrations were comparable in apes and humans, but were much lower in monkeys. This higher HDL in apes was predominantly in HDL_2. While the higher proportion in HDL_2 might be expected, unexpected was the lower HDL_3

(13-17 mg/dl) in apes than in humans. Even humans with low serum HDL cholesterol concentrations range from 25-35 mg/dl. Gorilla HDL_2 was similar to human HDL_2 in molecular weight (400,000 vs 358,000 (17), 400,000 (26), and 420,000 (8)), and density (1.077 vs 1.096 (17) and 1.090 g/ml (8)).

Properties of gorilla and rhesus HDL–In order that more detailed comparisons could be made, we further compared gorilla and rhesus monkey HDL as to the density distribution, peak density, peak flotation rate and molecular weights of different density fractions. All of these confirmed the striking difference between the properties of gorilla and rhesus HDL. Gorilla HDL was primarily (>70%) in the 1.10 g/ml density fraction, while only 30% of the rhesus HDL or human HDL_3 were found there. The low peak density of gorilla HDL (1.077 g/ml) suggested that it would be difficult to avoid cross-contamination with LDL. We carefully checked for its presence by agarose electrophoresis and analytical ultracentrifugation and found none. The low peak density was not likely due to aging of the sample since we found that aging of gorilla HDL for several months shifted the peak density to higher densities.

Monkey HDL properties–The second important finding was that rhesus and presumably other monkey HDL usually did not compare closely with human HDL_3 in density distribution or in composition. However, the comparative compositions of rhesus HDL and human HDL_3 were very similar in identical density fractions.

Number of components in monkey HDL–Rhesus were anomolous, usually having a single HDL component which was like human HDL_3 in molecular weight (209,000 vs 200,000), but had a lighter density (1.109 g/ml) overall than human HDL_3 (1.129 g/ml) and contained a lower % protein. It must be stressed that diet is extremely important. On a cholesterol diet, HDL_2 and HDL_3 become evident as separate peaks, although at high cholesterol serum levels (low HDL levels), HDL_2 disappears (7). The monkey chow diet is very low in fat and in cholesterol. Even under these conditions, a few rhesus monkeys produced an HDL having a clearly discernible HDL_2 and HDL_3, while a few had a fast floating HDL, having an S_f, 1.2 of 5-6, rather than the 4.6 usually observed. (HDL_2 S_f would be 6-8.) We observed further that the concentration of total HDL cholesterol was a reasonable predictor of the type of HDL which would be found. In general, monkeys with higher HDL cholesterol were more likely to show two easily discernible HDL peaks in the analytical ultracentrifuge. Mangabey monkeys differed in this regard. In the chow-fed animal, HDL_2 and HDL_3 could not be seen regardless of the concentration of HDL cholesterol, whereas in the cholesterol-fed state, even where HDL cholesterol was no higher than chow-fed animals, we frequently observed easily separable HDL_2 and HDL_3 (7).

Densities of rhesus HDL and human HDL_3–Rhesus HDL and human HDL_3 peaked at differing densities (1.109 and 1.129 g/ml). This was caused by human HDL_3 having a distribution shifted to higher densities with twice as much cholesterol in the >1.125 g/ml fraction as in the rhesus. Almost half of the <1.10 g/ml human HDL was HDL_2, since the human HDL samples contained 87% HDL_3 and 13% HDL_2. Thus the actual density spread (density heterogeneity) of human HDL_3 and rhesus HDL was not greatly different. The molecular weights of rhesus HDL and human HDL_3 were within 10% of each other between 1.11 and 1.13 g/ml densities. At 1.085 g/ml, both were 40% higher. Thus, at least in rhesus, the molecular size envelope was not symmetrical. In human, this was probably due to the HDL_2 present in our samples.

We found more HDL floating at density 1.125 g/ml for rhesus than other investigators. We found 83% floating, while Scanu et al. (18) found 66%. On a 36% (calories) corn oil diet, Portman et al. (19) found 37% floating. For cynomolgus monkeys, a species closely related to the rhesus, we found 71% floating at 1.125 g/ml. Assuming the corn oil diet to cause the lower HDL_2, those values also support the contention that monkeys have a less dense HDL than human HDL_3 and thus are often arbitrarily said to contain HDL_2 because of some HDL floating at density 1.125 g/ml. This is not necessarily incorrect, because HDL is heterogeneous, and the fraction floating at density 1.125 g/ml is a subpopulation of HDL, whatever name it is given. However, it is not a discontinuous HDL density fraction as HDL_2 is in humans.

Our composition data agrees in comparable density fractions with that of Anderson et al. (20), who reported human HDL was composed of HDL_{2b}, HDL_{2a} and HDL_3. Our molecular weight of the HDL_{2b} density fraction was much lower. This probably was because our monkeys were chow-fed and had little HDL_2. When cholesterol-fed, they develop an HDL_2 (7). Presumably, detailed study of this HDL would reveal HDL_{2a}-like properties in molecular weight in the 1.10-1.125 g/ml density gradient region. Indeed, studies on cholesterol-fed African green HDL which exhibited distinct

HDL_2 and HDL_3 peaks in density gradients revealed that the molecular weights and compositions of arbitrary density gradient subfractions corresponded closely to that of Anderson et al. (20) for human HDL_{2b}, HDL_{2a}, and HDL_3 (27).

Indeed the nomenclature of Anderson et al. (20) could have been used to describe HDL subfractions. For instance, gorilla HDL could be described as HDL_{2b} and HDL_{2a}, with very little HDL_3 (>1.125 g/ml density). Fless et al. (28) chose to term the two peaks they observed in density gradients as HDL_L and HDL_H, presumably since they do not match human HDL_2 and HDL_3 in densities and alter in densities upon cholesterol feeding. We chose to use the HDL_2 and HDL_3 terms, pointing out the comparisons and differences among species, while density fractionating HDL further so as also to compare similar density intervals among members of the major primate species.

The present comprehensive studies reveal many complexities in the distribution of serum HDL lipoproteins in the broadest comparative study thus far undertaken of sub-human primates. While our understanding of the metabolism of HDL has progressed in the last 10 years, the present work reveals that further detailed studies will be required, in perturbed states of cholesterol and fat feeding, before judgment can be made of the role of these substances in cholesterol and lipid homeostasis.

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REFERENCES

1. Rudel, L.L., and Morris, M.D. (1973) J. Lipid Res. 14, 364-366.
2. Burstein, M., and Samaille, J. (1960) Clin. Chim. Acta 5, 609.
3. Sardesai, V.M., and Manning, J.A. (1968) Clin. Chem. 14, 156-161.
4. Nobel, R.P. (1968) J. Lipid Res. 9, 693-700.
5. Nelson, C.A., and Morris, M.D. (1983) Lipids 18, 553-557.
6. Rudel, L.L.; Lee, J.A.; Morris, M.D.; and Felts, J.M. (1974) Biochem. J. 139, 89-95.
7. Nelson, C.A.; Greer, W.E.; and Morris, M.D. (1983) Biochem. Med. 29, 214-226.
8. Anderson, D.W.; Nichols, A.V.; Forte, T.M.; and Lindgren, F.T. (1977) Biochim. Biophys. Acta 493, 55-68.
9. Tall, A.R.; Blum, C.B.; Forester, G.P.; and Nelson, C.A. (1982) J. Biol. Chem. 257, 198-207.
10. Nelson, C.A.; Lee, J.A.; Brewster, M.; and Morris, M.D. (1974) Analyt. Biochem. 59, 69-74.
11. Hartree, E.F. (1972) Anal. Biochem. 48, 422-427.
12. Fiske, C.H., and Subbarow, Y. (1925) J. Biol. Chem. 66, 357-400.
13. Srinivasan, S.R.; McBride, J.R., Jr.; Radhakrishnamurthy, B.; and Berenson, G.S. (1974) Comp. Biochem. Physiol. 47B, 711-716.
14. Rudel, L.L., and Lofland, H.B. (1976) in Primates in Medicine (Goldsmith, E.I. and Moor-Jankowski, J., eds.) Vol. 9, pp. 224-266, S. Karger, Basel.
15. Nelson, C.A.; Casali, R.E.; and Morris, M.D. (1979) Biochem. Med. 22, 110-118.
16. Cheung, M.C., and Albers, J.J. (1979) J. Lipid Res. 20, 200-207.
17. Patsch, W.; Schonfeld, G.; Gotto, A.M., Jr.; and Patsch, J.R. (1980) J. Biol. Chem. 255, 3178-3185.
18. Scanu, A.M.; Edelstein, C.; Vitello, L.; Jones, R.; and Wissler, R. (1973) J. Biol. Chem. 248, 7648-7652.
19. Portman, O.W.; Alexander, M.; Neuringer, M.; Illingworth, D.R.; and Alam, S.S. (1981) J. Nutrition 111, 733-745.
20. Anderson, D.W.; Nichols, A.V.; Pan, S.S.; and Lindgren, F.T. (1978) Atherosclerosis 29, 161-179.
21. Reardon, M.F.; Sakai, H.; and Steiner, G. (1982) Atherosclerosis 2, 396-402.
22. Groot, P.H.E.; Jansen, H.; and Van Tol, A. (1981) FEBS Lett. 129, 269-272.
23. Shirai, K.; Barnhart, R.L.; and Jackson, R.L. (1981) Biochem. Biophys. Res. Commun. 100, 591-599.
24. Patsch, J.R.; Gotto, A.M., Jr.; Olivecrona, T.; and Elsenberg, S. (1978) Proc. Natl. Acad. Sci. USA 75, 4519-4523.
25. Nikkila, E.A.; Taskinen, M.-R.; and Kekki, M. (1978) Atherosclerosis 29, 497-501.
26. Hazelwood, R.N. (1958) J. Am. Chem. Soc. 80, 2152-2160.
27. Rudel, L.L.; Nelson, C.A.; and Weiss, K.R., manuscript in preparation. Reported at ASBC Meeting in New Orleans, 1982.
28. Fless, G.M.; Juhn, D.; Karlin, J.; Rubenstein, A.; and Scanu, A.M. (1984) Arteriosclerosis 4, 154-164.

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The Effects of Dietary Cholesterol on Blood and Liver Polyunsaturated Fatty Acids and on Plasma Cholesterol in Rats Fed Various Types of Fatty Acid Diet

Y.S. HUANG*, M.S. MANKU and **D.F. HORROBIN**, *Efamol Research Institute, P.O. Box 818, Kentville, Nova Scotia, Canada B4N 4H8*

ABSTRACT

Male rats were fed on a fat-free diet for 8 weeks and then switched to diets containing 10% hydrogenated coconut oil (HCO), safflower oil (SFO) or evening primrose oil (EPO). Half of each group was also given 1% of cholesterol in the diet. After 5 further weeks, plasma, red cell and liver fatty acids were measured in the various lipid fractions. Plasma and liver cholesterol also were estimated. In almost all fractions and on all three diets, feeding cholesterol led to accumulation of the substrates of desaturation reactions and to deficits of the products of these reactions. The results were consistent with inhibition of Δ-6, Δ-5 and Δ-4 desaturation of n-6 essential fatty acids. Since the diets were deficient in n-3 fatty acids, levels were very low but were also consistent with inhibition of desaturation. In contrast, cholesterol had relatively less consistent effects on 20:3n-9, suggesting that desaturation of n-9 fatty acids was less inhibited. Plasma cholesterol levels rose sharply in the HCO and SFO groups but not at all in the EPO group. EPO contains the product of Δ-6-desaturation, 18:3n-6, suggesting that conversion of linoleic acid to 18:3n-6 and possibly to further metabolites may be important for the cholesterol-lowering effect of polyunsaturates.
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INTRODUCTION

The addition of adequate amounts of polyunsaturated fatty acids (PUFA) to the diet of humans consistently lowers plasma cholesterol levels (1,2). The mechanism remains uncertain but the evidence is compelling enough to allow bodies such as the American Heart Association to recommend a general increase in dietary polyunsaturates (3).

While there is an immense literature on the effects of polyunsaturates on cholesterol, there is much less information on the effects of cholesterol on polyunsaturate metabolism. Although the possibility that cholesterol synthesis is reduced by PUFAs has been ruled out by many investigators (4-7), data reported by Bochenek et al. (8) have demonstrated that feedback inhibition by dietary cholesterol on hepatic cholesterol synthesis is greater when linoleic acid is fed with the cholesterol. Cholesterol fed to monkeys lowers total blood polyunsaturate levels (9). Feeding cholesterol to animals with sub-optimal essential fatty acid (EFA) intake leads to a striking exacerbation of the signs of EFA deficiency (10-12). This suggests that cholesterol may interfere with EFA metabolism or increase the need for EFAs. These indirect indications of a cholesterol effect on polyunsaturate metabolism have been followed up by direct measurements of individual fatty acids in some studies. Most investigations have looked only at linoleic and arachidonic acids, and a consistent finding is that arachidonic acid levels are reduced by cholesterol, while linoleic acid concentrations are unchanged or elevated (8,13-15). One report also noted an unusual elevation of dihomo-gamma-linolenic acid (20:3n-6), an intermediate in the pathway from linoleic acid to arachidonic acid (13). These studies suggest that cholesterol may reduce the efficiency of conversion of linoleic to arachidonic and possibly may also lower arachidonic acid levels by increasing utilization in the formation of cholesterol esters (8,13).

The present study aimed to examine further the effects of cholesterol on blood and liver fatty acid levels in animals fed a fat-free diet supplemented with saturated or unsaturated fats.

MATERIALS AND METHODS

Forty two male Sprague Dawley rats, weighing about 100 g, were maintained on a fat-free (FF) diet. The diet (TD 83231) was supplied by Teklad Test Diets (Madison, Wisconsin) and contained 66.8% sucrose, 22.2% vitamin-free casein, 5.6% cellulose, 4% mineral mix (AIN-76), 1.1% vitamin mix and 0.3% DL-methionine. After 8 weeks on this diet, animals showed growth retardation and scaly skin on feet and tail, indicating the onset of EFA deficiency. Animals were then allocated to six matched

*To whom correspondence should be addressed.

weight groups of 7 rats each and were fed for 5 further weeks with oil supplemented diets. To the FF diet was added 10% by weight of either hydrogenated coconut oil (HCO), safflower oil (SFO) or evening primrose oil (EPO). Half the 14 animals in each of the three groups were also given 1% by weight of cholesterol (CH) in the diet. The experiment was designed to deprive animals of EFAs and then to provide linoleate-rich oils with or without the addition of cholesterol, so that the interaction between dietary cholesterol and EFA could be examined with minimum complication from the endogenous EFA pool. HCO was virtually EFA free (contained less than 0.1% linoleic acid); SFO was rich in linoleic acid (79.5%), and EPO had about 9% of 18:3n-6 and 71% of linoleic acid, giving a total EFA content similar to that of SFO. The SFO and EPO supplemented animals were used for comparison of the effect of 18:3-n-6, while the HCO supplemented animals were used as the isocaloric controls.

All animals were kept in groups of two or three in plastic cages and had free access to food and water. At the end of the dietary experiment, animals were killed by exsanguination under light ether anesthesia between 8:00 and 10:00 a.m. Blood was collected via the abdominal aorta into a test tube containing ethylenediaminetetraacetate (EDTA) (1 mg/ml blood). Plasma was separated from erythrocytes by centrifugation. Livers were excised, rinsed in ice cold saline, blotted, weighed and frozen for lipid extraction.

Plasma and liver lipids were extracted by the method of Folch et al. (16), and erythrocyte lipids by that of Dodge and Phillips (17). Plasma cholesterol was measured enzymatically using a Cobas-Bio centrifugal analyzer (Hoffman-La Roche). Total liver cholesterol was measured after saponification of aliquots of liver lipid extracts and quantitated as trimethylsilane ether derivatives with 5-alpha-cholestane as internal standard by gas liquid chromatography (GLC) using 3% OV-1 on Gas Chrom Q (Applied Science Laboratories, State College, Pennsylvania) packed column. Free and ester cholesterols were separated by thin layer chromatography (TLC) and quantitated by GLC by the above method. Phospholipids and lipid fractions separated by TLC were methylated according to Morrison and Smith (18) and subjected to GLC for determination of fatty acid composition. A 10% Silar 10 C on Gas Chrom Q column was used with a Hewlett Packard 5880A machine with automated integration as described previously (19).

Statistical comparisons were made by Student's t-test.

RESULTS

At the end of the dietary experiment, the symptoms of EFA deficiency, scaly skin and rough fur, were significantly alleviated in animals fed SFO or EPO, but not in animals fed HCO.

Table 1 shows the body and liver weights of animals. Both SFO and EPO animals had similar body weights at the time of sacrifice, and these were not significantly affected by the cholesterol supplementation. The HCO animals had lower body weights which also were not affected by the cholesterol supplementation. Supplementation with cholesterol had no significant effect on liver weight in the HCO group but significantly increased the actual and relative liver weight of animals in SFO and EPO groups. Table 1 also shows the plasma and liver cholesterol levels. None of the diets had any effect on baseline cholesterol levels in the absence of dietary cholesterol. Dietary cholesterol substantially elevated plasma cholesterol in the HCO and SFO group but had only a small effect in the EPO group. In the liver, in the groups without added dietary cholesterol, total liver cholesterol in the EPO group was significantly lower than in the other two groups. In the groups with dietary cholesterol, liver cholesterol was higher in the EPO groups than in the other two groups. The excess cholesterol in the liver was mainly in the form of esters.

The fatty acid composition of plasma total phospholipids is shown in Table 2. Levels of n-3 fatty acids were generally low because of the diets and are not shown. The distribution of fatty acids in rats fed HCO diet reflects EFA deficiency with low levels of 18:2n-6 and 20:4-n-6 and a high level of 20:3n-9. Cholesterol feeding increased both 18:1n-9 and 20:3n-9 but decreased 22:5n-6. Supplementation with either SFO or EPO significantly raised the levels of 18:2n-6 and 20:4n-6 and also of other long chain n-6 fatty acids. Cholesterol supplementation decreased the proportions of 20:4-n-6 and other n-6 fatty acids, with the exceptions of 18:2n-6 and 20:3n-6, which were increased.

Table 3 shows the effect of diet with or without cholesterol supplementation on the percentage distribution of fatty acids of plasma cholesteryl esters. In HCO treated animals, CH supplementation significantly reduced the levels of 20:4n-6 and 22:5n-6. In SFO and EPO animals, CH feeding significantly elevated both 18:2n-6 and 20:3n-6 but reduced 20:4n-6 and 22:5n-6.

Effects of cholesterol feeding on fatty acid

TABLE 1

Effects of Dietary Cholesterol on Body and Liver Weights, Plasma and Liver Cholesterol Contents (Mean ± SEM)

	HCO		SFO		EPO	
	-CH	+CH	-CH	+CH	-CH	+CH
Body wt (g)	456 ± 18	490 ± 13	494 ± 13[b]	506 ± 10	498 ± 15[b]	507 ± 16
Liver wt (g)	16.0 ± 1.6	16.5 ± 0.9	13.7 ± 1.9	21.8 ± 1.0[a]	15.9 ± 0.9	20.4 ± 1.3[a]
% Body wt	3.5 ± 0.3	3.4 ± 0.1	3.8 ± 0.1	4.3 ± 0.1[a]	3.2 ± 0.2	4.0 ± 0.2[a]
Plasma CH (mg/dl)	48 ± 1.2	132 ± 11.0[a]	46 ± 4.0	116 ± 5.2[a]	53 ± 3.1	63 ± 4.9[a,b]
Liver CH (mg/g)	2.3 ± 0.2	13.1 ± 4.9[a]	2.6 ± 0.3	11.4 ± 1.1[a]	1.6 ± 0.1[b]	14.7 ± 1.5[a]
Free (mg/g)	1.2 ± 0.2	3.2 ± 0.6[a]	1.8 ± 0.1[b]	0.9 ± 0.1[a,b]	1.2 ± 0.1	1.7 ± 0.2[a,b]
Ester (mg/g)	1.1 ± 0.2	9.9 ± 2.9[a]	0.8 ± 0.2[b]	10.5 ± 0.8[a]	0.5 ± 0.1[b]	13.1 ± 1.4[a]

For details, see Materials and Methods.
[a]Significantly different from no cholesterol group at $p < 0.01$ or better.
[b]Significantly different from HCO group at $p < 0.05$ or better.

TABLE 2

Effects of Dietary Cholesterol on Fatty Acid Composition of Plasma Phospholipids

	HCO		SFO		EPO	
Fatty Acids	-CH	+CH	-CH	+CH	-CH	+CH
16:0	20.6 ± 1.1[a]	20.3 ± 1.0	20.4 ± 1.1	19.8 ± 1.3	19.5 ± 1.8	21.1 ± 2.4
16:1n-7	4.7 ± 1.8	4.7 ± 0.9	1.3 ± 1.5	2.9 ± 0.7	1.0 ± 0.8	1.0 ± 0.5
18:0	19.1 ± 2.5	17.4 ± 3.0	11.0 ± 1.5	9.0 ± 2.5	14.0 ± 2.1	12.3 ± 3.7
18:1n-9	19.1 ± 1.5	22.1 ± 2.5[b]	6.0 ± 1.4	9.3 ± 1.0[c]	6.6 ± 1.4	9.6 ± 0.8[c]
20:3n-9	20.5 ± 1.4	22.7 ± 1.6[b]	—	0.7 ± 0.2	—	—
18:2n-6	4.4 ± 1.3	3.6 ± 0.7	16.4 ± 2.5	27.0 ± 3.6[c]	14.5 ± 1.5	19.4 ± 2.1[c]
18:3n-6	—	—	1.1 ± 0.5	—	0.5 ± 0.1	0.6 ± 0.1
20:3n-6	—	—	0.6 ± 0.1	2.8 ± 1.1[c]	0.8 ± 0.4	2.6 ± 0.8[c]
20:4n-6	6.4 ± 0.9	5.3 ± 0.9	37.4 ± 3.9	25.7 ± 5.1[c]	38.9 ± 4.4	31.7 ± 2.0[c]
22:4n-6	0.2 ± 0.07	0.2 ± 0.04	0.7 ± 0.3	0.7 ± 0.3	0.9 ± 0.1	0.5 ± 0.1[b]
22:5n-6	1.8 ± 0.4	1.3 ± 0.3[b]	3.6 ± 1.0	0.9 ± 0.3[c]	3.2 ± 0.5	1.0 ± 0.3[c]

For details, see Materials and Methods.
Data are expressed as % by weight of total fatty acids.
[a]Each figure represents the mean ± SD of 7 animals.
[b,c]Statistical comparisons are for each diet with and without cholesterol by Student's t-test at levels of 0.05 and 0.01 or better.

TABLE 3

Effects of Dietary Cholesterol on Fatty Acid Composition of Plasma Cholesterol Esters

Fatty Acids	HCO		SFO		EPO	
	-CH	+CH	-CH	+CH	-CH	+CH
16:0	7.6 ± 3.3[a]	7.4 ± 5.9	1.4 ± 1.1	1.8 ± 0.9	7.0 ± 1.9	8.9 ± 1.1
16:1n-7	9.2 ± 6.0	18.3 ± 6.7[b]	1.7 ± 1.2	9.0 ± 3.0[c]	2.2 ± 1.0	8.4 ± 5.9[c]
18:0	1.3 ± 0.6	3.3 ± 3.4	0.5 ± 0.1	1.3 ± 0.9	1.0 ± 0.5	1.0 ± 0.1
18:1n-9	9.2 ± 6.7	31.4 ± 10.0[c]	2.6 ± 2.7	4.2 ± 1.1	6.0 ± 1.5	21.0 ± 3.4[c]
20:3n-9	37.6 ± 10.4	14.5 ± 6.5[c]	—	—	—	—
18:2n-6	9.4 ± 4.7	13.0 ± 7.9	15.0 ± 7.1	37.9 ± 7.3[c]	14.1 ± 2.8	26.9 ± 4.4[c]
18:3n-6	—		1.0 ± 0.2	1.5 ± 0.3	2.8 ± 0.6	1.9 ± 0.4
20:3n-6		—	0.2 ± 0.2	1.7 ± 1.1[b]	tr	1.2 ± 0.3
20:4n-6	21.4 ± 8.1	7.0 ± 1.2[c]	70.9 ± 11.6	39.5 ± 8.9[c]	66.4 ± 5.6	31.9 ± 5.4[c]
22:4n-6			—		—	—
22:5n-6	1.0 ± 0.3	0.5 ± 0.2[b]	1.0 ± 0.2	0.5 ± 0.2[c]	1.3 ± 0.2	tr

For details, see Materials and Methods.
Data are expressed as % by weight of total fatty acids.
[a]Each figure represents the mean ± SD of 7 animals.
[b,c]Statistical comparisons are for each diet with and without cholesterol by Student's t-test at levels of 0.05 and 0.01 or better.

composition in liver phospholipids are shown in Table 4. In the HCO group, cholesterol feeding reduced 22:5n-6. In the SFO and EPO groups, the proportions of 18:2n-6 and 20:3n-6 were increased, while those of 20:4n-6 and 22:5n-6 all were decreased significantly.

In liver CE (Table 5), the cholesterol feeding significantly decreased all n-6 fatty acids in the HCO group. Cholesterol feeding also reduced the proportions of 20:4n-6, 22:4n-6 and 22:5n-6, but increased those of 18:2n-6 and 20:3n-6 in the EPO group.

PC and sphingomyelin are the two major phospholipid fractions in red blood cell membranes readily exchangeable with plasma phospholipids (20). Since sphingomyelin contains mainly saturated or monounsaturated fatty acids, dietary supplementation of PUFAs did not significantly affect the fatty acid pattern of sphingomyelin. Therefore, in this study, only fatty acid of red blood cell-phosphatidyl choline (RBC-PC) are shown in Table 6. Cholesterol feeding had no significant effect on the RBC fatty acid pattern in the HCO group. However, significant increases of 18:2n-6 and 20:3n-6 and decreases of 20:4n-6 and 22:5n-6 in both the SFO and EPO groups occurred when the cholesterol diet was given. 22:4n-6 also was found decreased in the SFO group treated with cholesterol.

Plasma and liver triglycerides contained very low levels of 20:3n-6 and 20:4n-6. These results are not presented but are available for inspection. Cholesterol feeding affected the fatty acid patterns in these two fractions with trends similar to those found in phospholipids and cholesteryl esters.

The changes in essential fatty acid levels in response to feeding cholesterol were similar in all three diet groups and can be summarized as follows:

1. EFA levels were naturally very low in the HCO group, and n-3 EFA levels were low in all groups because of their absence from the diet.

2. Concentrations of 18:2n-6 were consistently elevated, especially in the SFO and EPO groups.

3. 18:3n-6 was present in only some fractions in trace amounts and was slightly elevated in the EPO group.

4. 20:3n-6 was undetectable in most fractions in the HCO group. In the SFO and EPO groups it was consistently elevated by cholesterol feeding. The rise was particularly striking in liver triglycerides in the EPO group (3.1 ± 0.5 vs 0.9 ± 0.3 mg/100 mg total fatty acids present).

5. 20:4n-6 was present only in small quantities in the HCO group but in much larger amounts in the SFO and EPO groups. Its levels were consistently reduced in all fractions by cholesterol feeding. The fall tended to be less in the EPO group than in the SFO group.

6. 22:4n-6 and 22:5n-6 were present in low concentrations in most fractions in the SFO and EPO groups and also consistently fell with cholesterol feeding.

DISCUSSION

These results leave little doubt that, as hinted at in many previous studies (8-15), cholesterol has a profound effect on the metabolism of n-6 EFAs. 18:2 and 20:3n-6 are consistently elevated and 20:4n-6 is consistently reduced by cholesterol feeding. 22:4n-6 also tends to be reduced, but 22:5n-6 is lowered to a much greater extent. The ratios of substrates to products at all three desaturation steps (Δ-6, Δ-5, Δ-4), were increased. This suggests either that cholesterol inhibits desaturation or that, less likely, in all fractions in the plasma, red cells and liver it selectively enhances incorporation of substrates for desaturation. Since the rats were deficient in n-3 EFAs, little information was provided on n-3 metabolism although there certainly was reduced formation of the last major metabolite, 22:6n-3 (not shown, results available from the authors).

n-3, n-6 and n-9 fatty acids compete with one another for desaturation systems and it is commonly believed that the same enzymes are involved with all three series (21,22). The accumulation of 20:3n-9 is a widely used marker of EFA deficiency, since its formation from 18:1n-9 is readily inhibited by both n-3 and n-6 EFAs (21). Although the present studies show clear modification of n-6 metabolism by cholesterol, 20:3n-9 levels were unchanged or elevated by cholesterol, except in the cholesterol ester fraction in the HCO group. This suggests that cholesterol may modify 18:1n-9 metabolism less than that of the n-6 fatty acids.

The data suggest that cholesterol leads to less efficient metabolism of 18:2n-6 along the essential fatty acid pathway. There also may be increased utilization of arachidonic acid as a contributory factor to the low levels of 20:4n-6 (13), but such raised utilization cannot be the whole explanation. If it were, one would expect to see reduced levels of the arachidonate precursors 20:3n-6 and 18:2n-6 instead of the raised concentrations actually observed. In humans there is one small study which provides direct evidence for a reduced metabolism of linoleic acid in patients with elevated blood lipid levels (23). When ^{14}C-1-linoleic acid was admi-

TABLE 4

Effects of Dietary Cholesterol on Fatty Acid Composition of Liver Total Phospholipids

Fatty Acids	HCO		SFO		EPO	
	-CH	+CH	-CH	+CH	-CH	+CH
16:0	16.6 ± 0.9[a]	17.7 ± 0.9	17.3 ± 0.8	15.9 ± 0.3[b]	18.1 ± 1.1	17.1 ± 0.4
16:1n-7	4.8 ± 1.2	4.5 ± 0.5	0.9 ± 0.3	2.9 ± 0.6[c]	1.7 ± 0.5	2.3 ± 0.6
18:0	19.1 ± 0.6	21.5 ± 1.0[b]	20.5 ± 1.8	14.8 ± 1.2[c]	17.9 ± 1.4	14.0 ± 1.9[c]
18:1n-9	19.2 ± 1.7	18.8 ± 1.8	7.8 ± 1.4	12.9 ± 1.1[c]	9.5 ± 1.3	12.9 ± 1.6[c]
20:3n-9	19.8 ± 1.2	20.0 ± 1.2	0.3 0.1	0.9 ± 0.2[c]	0.3 ± 0.04	0.5 ± 0.08[b]
18:2n-6	2.8 ± 0.6	3.0 ± 0.3	10.3 ± 0.7	18.7 ± 2.2[c]	9.5 ± 0.8	17.1 ± 2.1[c]
18:3n-6	—	—	—	—	—	—
20:3n-6	—	—	0.5 ± 0.2	3.3 ± 1.2[c]	0.8 ± 0.3	2.7 ± 0.8[c]
20:4n-6	10.9 ± 1.7	8.5 ± 1.0	34.3 ± 0.7	26.4 ± 3.2[c]	35.6 ± 1.0	30.3 ± 2.3[c]
22:4n-6	—	—	0.9 ± 0.2	0.7 ± 0.1	1.0 ± 0.09	0.6 ± 0.09[c]
22:5n-6	2.8 ± 0.6	1.7 ± 0.5[b]	5.2 ± 1.0	1.6 ± 0.5[c]	4.8 ± 0.9	1.2 ± 0.5[c]

For details, see Materials and Methods.
Data are expressed as % by weight of total fatty acids.
[a]Each figure represents the mean ± SD of 7 animals.
[b,c]Statistical comparisons are for each diet with and without cholesterol by Student's t-test at levels of 0.05 and 0.01 or better.

TABLE 5

Effects of Dietary Cholesterol Feeding on Fatty Acid Composition of Liver Cholesterol Esters

Fatty Acids	HCO		SFO		EPO	
	-CH	+CH	-CH	+CH	-CH	+CH
16:0	21.6 ± 2.2[a]	21.8 ± 1.2	21.8 ± 3.5	13.1 ± 1.3[c]	30.5 ± 11.4	10.9 ± 1.6[c]
16:1n-7	17.7 ± 2.4	15.8 ± 1.7	4.6 ± 2.7	20.9 ± 2.4[c]	6.0 ± 2.5	19.6 ± 4.4[c]
18:0	3.6 ± 0.6	3.8 ± 0.6	8.3 ± 2.2	1.5 ± 0.7[c]	13.1 ± 9.3	0.6 ± 0.2[c]
18:1n-9	50.0 ± 5.0	53.1 ± 1.6	22.2 ± 5.0	34.0 ± 2.5[c]	26.3 ± 7.4	31.0 ± 1.6
20:3n-9	2.8 ± 0.5	0.9 ± 0.2[c]	0.5 ± 0.1	0.6 ± 0.1	--	
18:2n-6	2.2 ± 0.3	1.4 ± 0.3[c]	23.8 ± 2.8	23.6 ± 2.2	14.3 ± 7.4	29.1 ± 5.3[c]
18:3n-6	–		--		2.3 ± 0.8	tr
20:3n-6	–	–	0.7 ± 0.2	1.0 ± 0.2	1.0 ± 0.3	1.8 ± 0.2[b]
20:4n-6	0.9 ± 0.3	0.3 ± 0.08[b]	13.0 ± 2.6	3.0 ± 1.4[c]	13.8 ± 2.3	3.5 ± 0.6[c]
22:4n-6	–	--	0.7 ± 0.5	0.2 ± 0.09[c]	1.4 ± 1.7	0.1 ± 0.02[c]
22:5n-6	0.2 ± 0.08	0.1 ± 0.04[b]	1.2 ± 0.4	0.3 ± 0.2[c]	0.7 ± 0.1	0.1 ± 0.02[c]

For details, see Materials and Methods.
Data are expressed as % by weight of total fatty acids.
[a]Each figure represents the mean ± SD of 7 animals.
[b,c]Statistical comparisons are for each diet with and without cholesterol by Student's t-test at levels of 0.05 and 0.01 or better.

TABLE 6

Effects of Dietary Cholesterol on Fatty Acid Composition of Red Cell Phosphatidyl-Choline

Fatty Acids	HCO		SFO		EPO	
	-CH	+CH	-CH	+CH	-CH	+CH
16:0	39.4 ± 1.9[a]	37.9 ± 1.3	39.1 ± 2.6	38.1 ± 0.7	40.8 ± 1.6	40.5 ± 1.5
16:1n-7	4.7 ± 0.8	5.4 ± 1.5	0.6 ± 0.2	1.6 ± 0.5[c]	0.8 ± 0.3	1.4 ± 0.5[b]
18:0	10.5 ± 1.6	9.9 ± 1.0	15.5 ± 1.4	12.9 ± 0.6[b]	10.6 ± 2.1	10.1 ± 1.4
18:1n-9	19.6 ± 0.8	23.2 ± 1.6[b]	8.5 ± 1.1	10.7 ± 0.9[b]	6.8 ± 0.6	9.1 ± 1.3[c]
20:3n-9	14.4 ± 1.2	14.0 ± 2.0	0.3 ± 0.1	0.6 ± 0.3	—	0.3 ± 0.07
18:2n-6	2.7 ± 0.2	2.9 ± 0.5	13.8 ± 0.9	19.3 ± 1.2[c]	13.8 ± 1.2	17.3 ± 2.2[c]
18:3n-6	—	—	—	—	0.3 ± 0.08	0.4 ± 0.07
20:3n-6	—	—	0.5 ± 0.2	2.0 ± 0.9[b]	0.7 ± 0.1	2.6 ± 0.8[c]
20:4n-6	6.5 ± 0.4	5.0 ± 0.6	19.2 ± 2.3	13.2 ± 1.9[c]	24.1 ± 1.9	16.8 ± 1.1[c]
22:4n-6	—	—	0.5 ± 0.1	0.3 ± 0.8[b]	—	—
22:5n-6	1.0 ± 0.2	0.9 ± 0.2	1.9 ± 0.4	0.6 ± 0.2[c]	1.6 ± 0.4	0.7 ± 0.2[b]

For details, see Materials and Methods.
Data are expressed as % by weight of total fatty acids.
[a]Each figure represents the mean ± SD of 7 animals.
[b,c]Statistical comparisons are for each diet with and without cholesterol by Student's t-test at levels of 0.05 and 0.01 or better.

nistered to normals and to patients with high blood lipids, approximately double the amount of radioactivity appeared in 20:3n-6 and 20:4-n-6 in the normals as in the hyperlipidemics (23).

The groups with and without cholesterol represent two extremes of cholesterol metabolism. In the absence of dietary cholesterol all available cholesterol must be synthesized endogenously. In the presence of a massive excess of dietary cholesterol, endogenous cholesterol synthesis will be maximally suppressed and the majority of the cholesterol will be from the exogenous source. In the absence of cholesterol in the diet, while plasma cholesterol concentrations were similar in all three groups, total liver cholesterol was significantly lower in the EPO group. This suggests that 18:3n-6 or one of its metabolites is able to reduce endogenous cholesterol synthesis. In the cholesterol-fed groups, in contrast, total liver cholesterol was highest and plasma cholesterol lowest in the EPO group. This suggests that EPO is able to stimulate the transport of cholesterol from the plasma into the liver. Thus there appear to be two separate effects, a suppression of cholesterol synthesis and an enhancement of clearance of cholesterol from the plasma. The interactions of these two need to be studied further in situations in which the balance between endogenous cholesterol synthesis and exogenous cholesterol supplies are more similar to those in real life.

Since the only major dietary difference between the SFO and EPO groups was the presence of 18:3n-6 in the latter, this fatty acid presumably was the cause of the plasma cholesterol-lowering action and the effects on the liver. This is consistent with the observation that EPO and arachidonate seem to be much more potent than linoleic acid-containing oils in lowering cholesterol levels in humans and animals (24-26). Neither the human nor the rat studies provide any evidence as to whether the active metabolite is the same for both effects or whether it is 18:3n-6, 20:3n-6, 20:4n-6, a cyclooxygenase metabolite, a lipoxygenase metabolite or some combination. In the present studies, 18:3n-6, 20:3n-6 and 20:4n-6 concentrations were in most fractions higher in the EPO group than the SFO group.

Since a metabolite of 18:2n-6 lowers plasma cholesterol, while feeding cholesterol inhibits desaturation of linoleic acid, there is the suggestion of a feedback relationship between the effects of polyunsaturates on cholesterol and of cholesterol on polyunsaturate metabolism. One possibility, consistent with the currently known facts, is that a metabolite of 18:2n-6 stimulates the transfer of plasma cholesterol into liver cells, either via LDL-receptors or via one of the alternative routes (27). This will raise the hepatic intracellular cholesterol concentrations. This cholesterol will then progressively inhibit desaturation of linoleic acid, leading to reduced concentrations of active metabolites and hence to a reduced inflow of cholesterol from plasma. Such a system would help to regulate the plasma and intracellular cholesterol levels.

REFERENCES

1. Hegsted, D.M.; McGancy, R.B.; Myers, M.L., and Stare, F.M. (1965) Am. J. Clin. Nutr. 17, 281-295.
2. Keys, A.; Anderson, J.T., and Grande, F. (1957) Lancet ii, 959-966.
3. Grundy, S.M.; Bilheimer, D.; Blackburn, H.; Brown, W.V.; Kwiterovich, P.O.; Maltson, F.; Schonfeld, G., and Weidman, W.H. (1982) Circulation 65, 839A-854A.
4. Franz, I.D. and Carly, J.B. (1961) Proc. Soc. Exp. Biol. Med. 106, 800-801.
5. Grundy, S.M. and Ahrens, E.H. (1970) J. Clin. Invest. 49, 1135-1152.
6. Nestel, P.J.; Havenstein, N.; Scott, T.W., and Cook, L.J. (1974) Aust. N.Z.J. Med. 4, 497-501.
7. Illingworth, D.R. and Sandberg, E.E. (1981) Biochem. Soc. Trans. 9, 49.
8. Bochenek, W. and Rodgers, J.B. (1978) Biochim. Biophys. Acta 528, 1-16.
9. Portman, O.W.; Pinter, K., and Hayashida, T. (1959) Am. J. Clin. Nutr. 7, 63-69.
10. Peifer, J.J. and Holman, R.T. (1955) Arch. Biochem. Biophys. 57, 520-521.
11. Holman, R.T. and Peifer, J.J. (1960) J. Nutr. 70, 411-417.
12. Gambal, D. and Quackenbush, F.W. (1960) J. Nutr. 70, 497-501.
13. Morin, R.J.; Bernick, S.; Mean, J.F., and Alfin-Slater, R.B. (1962) J. Lipid Res. 3, 432-438.
14. Ostwald, R. and Shannon, A. (1964) Biochem. J. 91, 146-154.
15. Crocker, P.J.; Fitch, M., and Ostwald, R. (1979) J. Nutr. 109, 927-938.
16. Folch, J.; Lees, M., and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497-509.
17. Dodge, J.T. and Phillips, G.B. (1967) J. Lipid Res. 8, 667-675.
18. Morrison, W.R. and Smith, L.M. (1964) J. Lipid Res. 5, 600-608.
19. Manku, M.S.; Horrobin, D.F.; Huang, Y.S., and Morse, N. (1983) Lipids 18, 906-908.
20. Reed, C.F. (1968) J. Clin. Invest. 47, 749-760.
21. Holman, R.T. (1964) Fed. Proc. 23, 1062-1066.
22. Bernert, J.T. and Sprecher, H. (1975) Biochim. Biophys. Acta 398, 354-363.
23. Nichaman, M.Z.; Olson, R.E., and Sweeley, C.C. (1967) Am. J. Clin. Nutr. 20, 1070-1083.
24. Horrobin, D.F. and Manku, M.S. (1983) Lipids 18, 558-562.
25. Peifer, J.J. (1966) J. Nutr. 88, 351-358.
26. Kingsbury, K.J.; Morgan, D.M.; Aylcott, C., and Emmerson, R. (1961) Lancet i, 739-742.
27. Goldstein, J.L.; Kita, T., and Brown, M.S. (1983) N. Engl. J. Med. 309, 288-296.

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Milk Fat Structure of a Patient With Type 1 Hyperlipidemia

J.J. MYHER,[a] A. KUKSIS[a,*] and G. STEINER,[b] [a]*Banting and Best Department of Medical Research and* [b]*Department of Medicine, University of Toronto, Toronto, Ontario M5G 1L6, Canada*

ABSTRACT

Structural analyses were performed on milk fat samples obtained 3-10 days postpartum from a lactating patient with primary Type 1 hyperlipidemia. The milk triacylglycerols contained 3-7% C_{10}, 14-21% C_{12}, 20-30% C_{14}, 22-26% C_{16} and 20-30% C_{18} (largely oleic) acids. Gas liquid chromatographic (GLC) analyses of the X-1,3- and X-1,2-diacylglycerols on polar siloxane columns showed a markedly non-random association of acyl chains. Stereospecific analyses indicated that the short chain length fatty acids were confined essentially to the sn-3-position of the triacylglycerol molecule. Furthermore, these acids were largely absent from the phosphatidylcholines and the endogenous sn-1,2-diacylglycerols of the milk fat. It is concluded that the short chain fatty acids are incorporated into the milk triacylglycerols during the final stage of biosynthesis via the phosphatidic acid pathway, and that the overall fatty acid distribution is consistent with the 1-random 2-random 3-random hypothesis. *Lipids* 19:673-682, 1984.

INTRODUCTION

We have reported previously (1) on the unusual composition of the milk fat of a lactating patient with a primary Type 1 hyperlipidemia characterized by a congenital deficiency of lipoprotein lipase. The previous data (1) suggested that the plasma lipids could not be taken up by the mammary gland. This results in a replacement of much of the plasma long chain-length fatty acids by the short and medium chain-length acids synthesized de novo in the mammary gland (2). Due to this substitution the molecular weight distribution of the milk fat triacylglycerols of this patient assumes the appearance of the distribution of the milk fat of ruminants and horses, which also incorporate much short and medium-chain acid into their milk triacylglycerols.

Since ruminant milk fats are known to possess characteristic structure in regards to both molecular association and positional distribution of the short chain acids (3-5), we have undertaken detailed analyses of the structure of the abnormal human milk fat in order to determine if the similarity with the ruminant milk fat also extends to the stereochemical structure of the triacylglycerols. The results indicate that the influx of the C_{10} and C_{12} fatty acids is confined largely to the sn-3-position of the triacylglycerols, in a close correspondence to the distribution of these acids in horse milk triacylglycerols.

MATERIALS AND METHODS

The milk samples were collected at 4-8 hr intervals 3-10 days postpartum as previously described (1). Each sample was approximately 10 ml. All samples were cooled to 2 C immediately after collection and were kept under refrigeration until analyzed. Total lipids were extracted (6) and mono-, di- and triacylglycerols were separated by thin layer chromatography (TLC), as described elsewhere (5). The trimethylsilyl (TMS) ethers of the X-1,2- and X-1,3-diacylglycerols were prepared and separately resolved according to molecular weight and degree of unsaturation using polar cyanopropylphenylsiloxane columns as previously described (7). Random degradation of triacylglycerols was performed by Grignard reaction (8). Stereospecific analyses were done on the total sample of the triacylglycerols by the method of Brockerhoff (9). The carbon number profiles of the sn-2,3-diacylglycerol moieties of the triacylglycerols were obtained from the sn-2,3-diacylphosphatidylphenols by pyrolysis in the hot (310 C) metal injector port of the gas chromatograph as previously reported (4). Fatty acid analyses were done as outlined elsewhere (5). The analytical standards and general methods of analysis were as described (10). In addition to the abnormal human milk samples, a sample of horse milk (11) was analyzed as a reference material.

RESULTS AND DISCUSSION

The general composition and interrelationships between the plasma and milk lipids of the subject with Type 1 hyperlipidemia have been described elsewhere (12), where the differences between normal milk and milk of the patient also were pointed out. In the present study we have examined in detail the structure of the triacylglycerols of the abnormal milk fat and

*To whom correspondence should be addressed.

have compared it to that of mare's milk to which it bears a striking resemblance.

Composition of Fatty Acids

Table 1 gives the fatty acid composition of several of the milk samples collected from the patient 3-10 days postpartum in comparison to that of a sample of horse milk triacylglycerols. It is seen that the different abnormal human milk samples are similar in their fatty acid composition and are characterized by the presence of a high proportion of C_{10}-C_{14} saturated fatty acids and small amounts of linoleic and the higher unsaturated fatty acids. Oleic acid is the major unsaturated fatty acid and accounts for about 20% of the total. These values differ markedly from those of normal human milk, which contains much less short and medium chain fatty acid and more polyunsaturated fatty acids (13). Mothers on high carbohydrate/low fat diets previously have been reported (14, 15) to produce milk enriched with medium chain length fatty acids. While the nature of our patient's diet (70% carbohydrate, 10% fat) might have been partly responsible for the observed milk fat composition, the proportions of the medium chain fatty acids in her milk are clearly outside the range which is reached merely with dietary manipulation. Furthermore, the changes in the fatty acid composition seen during the course of a single day are obviously outside those characterizing a normal diurnal variation (16). Animal studies have shown (17) that the mammary gland synthesizes medium chain length fatty acids in response to a shortage of unsaturated acids in the diet or in the blood stream. This may serve the purpose of decreasing the melting point of the largely saturated fat and increasing its solubility in the aqueous medium.

Molecular Association of Fatty Acids

Figure 1 represents the GLC profiles of the milk fat triacylglycerols as recorded for the samples taken on successive days of lactation. The initial milk sample was collected on the third day postpartum, and its triacylglycerol elution pattern shows some resemblance to that of normal human milk fat (13). However, by day 9 (Fig. 1B) postpartum the triacylglycerol pattern has become greatly distorted by the presence of high proportions of the medium chain triacylglycerols. As lactation progresses, the proportion of the short and medium chain length fatty acids (Table 1) increases further; this is reflected in the carbon number profile (Table 2).

Table 2 gives the quantitative carbon number distribution in the abnormal milk fat samples along with the carbon number distribution for the milk triacylglycerols of a normal subject and of the sample of horse milk. The closely similar distributions of the carbon numbers between the horse milk triacylglycerols and the triacylglycerols of some of the abnormal human milk samples are due to corresponding similarities in the carbon number distribution of the component fatty acids in these samples, as well

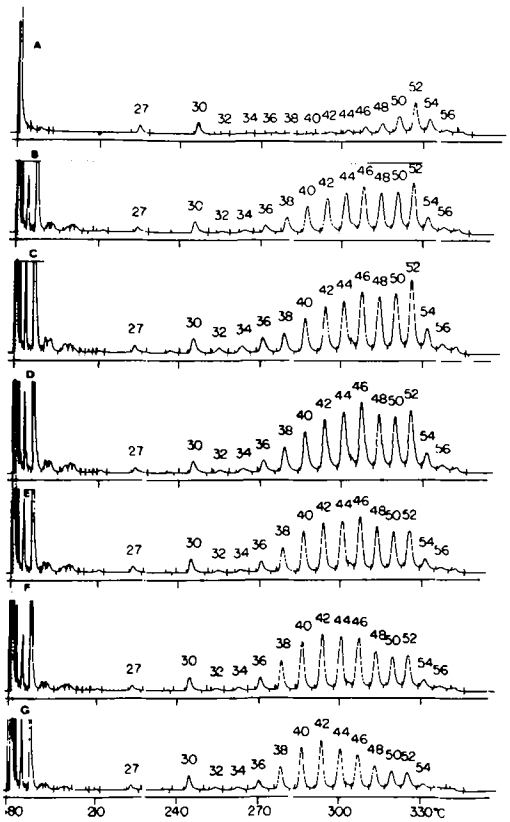

FIG. 1. Gas-liquid chromatographic resolution of the triacylglycerols of the milk fat of a patient with Type 1 hyperlipidemia. A, day 3 (2:30 p.m.); B, day 9 (7:50 a.m.); C, day 9 (11:30 a.m.); D, day 9 (4:00 p.m.); E, day 9 (8:00 p.m.); F, day 9 (11:30 p.m.); G, day 10 (8:00 a.m.). Peak 27, TMS ether of cholesterol; Peak 30, tridecanoylglycerol internal standard; Peaks 32-56, triacylglycerols with a total of 32-56 acyl carbons. Instrument: Hewlett-Packard Model 5700 gas chromatograph equipped with an unheated on-column injector, dual columns, differential electrometer, electronic peak area integrator and an automatic sample injector. Column: stainless steel tube, 50 cm × 2 mm I.D., packed with 3% OV-1 on Gas Chrom Q (100-120 mesh). Carrier gas, nitrogen at 80 ml/min. Detector temperature, 350 C. Column temperature program, 180 to 350 C at 8 C/min. Sample: 1 μl of the lipid in petroleum ether. Attenuation, 1/100 full sensitivity. Vertical lines intersecting the base line are the event marks of the peak area integrator.

TABLE 1

Fatty Acid Composition of Milk Triacylglycerols from a Patient with Type 1 Hyperlipidemia Compared to that of a Normal Subject and of a Horse

Fatty acids	Patient[a]							Normal subject (13)	Horse
	3d	9d1	9d2	9d3	9d4	9d5	10d		
	Moles %								
8:0									2.0
10:0		3.2	3.5	5.5	7.0	3.1	4.1	1.6	3.8
12:0	5.8	14.9	14.6	16.9	13.8	18.0	21.3	6.4	4.7
14:0	8.9	21.6	22.0	22.2	24.8	27.5	29.9	7.0	6.0
16:0	27.8	26.0	26.2	24.3	25.0	23.6	21.8	23.0	19.9
16:1ω7	2.7	1.5	1.4	1.9	1.8	1.4	0.7	3.7	5.8
18:0	5.7	5.9	5.5	5.5	4.6	5.8	4.7	5.7	1.0
18:1ω9	39.6	20.9	22.2	19.5	18.9	16.9	13.5	31.3	22.1
18:2ω6	7.6	4.1	3.5	3.1	2.7	2.9	2.8	16.6	16.3
18:3ω3									16.4
20:1ω9	0.8	0.8	0.6	0.6	1.0	0.2	0.8	0.7	
20:2ω6	0.6	0.4		0.1				1.0	
20:4ω6	0.1	0.2						0.6	
Other								0.8	

[a]Times of sampling: 3d, day 3 (2:30 p.m.); 9d1, day 9 (7:50 a.m.); 9d2, day 9 (11:30 a.m.); 9d3, day 9 (4:00 p.m.); 9d4, day 9 (8:00 p.m.); 9d5, day 9 (11:30 p.m.); 10d, day 10 (8:00 a.m.).

TABLE 2

Carbon Number Distribution of Milk Triacylglycerols from a Patient with Type 1 Hyperlipidemia Compared to that of a Normal Subject and of a Horse

Carbon number	Patient[a]							Normal subject (13)	Horse
	3d	9d1	9d2	9d3	9d4	9d5	10d		
	Mole %								
32	0.6	0.7	1.8	0.8	0.8	0.7	1.2	–	–
34	0.7	0.9	2.2	1.3	1.0	1.0	1.3	–	1.3
36	1.2	2.4	4.4	3.5	3.0	3.5	3.6	–	2.5
38	1.2	5.0	5.1	6.9	6.5	7.8	8.5	–	4.1
40	0.8	8.3	8.4	10.9	11.6	12.5	14.7	0.9	4.9
42	2.5	11.4	12.0	14.6	13.5	15.1	17.4	2.1	7.6
44	3.0	12.7	13.3	15.5	13.8	15.5	14.8	4.7	10.9
46	7.1	14.3	12.2	13.4	14.6	13.7	13.1	8.2	9.9
48	11.2	11.7	10.9	10.3	11.0	9.9	8.7	11.6	8.4
50	19.8	12.0	11.6	9.5	9.7	8.4	7.0	16.8	12.8
52	31.5	14.0	13.2	10.4	10.2	8.5	6.4	34.0	22.7
54	16.7	4.6	3.4	2.1	3.0	2.6	2.1	16.7	15.0
56	3.5	1.7	0.9	0.4	1.1	0.9	0.9	3.6	–
58	0.5	0.5						1.2	–

[a]Times of sampling as in Table 1.

as to similarities in the molecular association.

A more direct indication of the molecular association of fatty acids in the triacylglycerols is obtained by comparing the carbon number distribution in the sn-1,2(2,3)- and in the X-1,3-diacylglycerols derived from the milk fat triacylglycerols by Grignard degradation. Table 3 gives the quantitative distribution of the carbon numbers for the two types of diacylglycerols from abnormal milk samples as obtained by GLC analysis of the corresponding TMS ethers on non-polar columns. It is seen that both the X-1,3-diacylglycerols and the sn-1,2(2,3)-diacylglycerols contain significant quantities of medium chain-length species. Furthermore, both diacylglycerol types contain higher proportions of the shorter chain and lower proportions of the longer chain diacylglycerol moieties than would be predicted by the random calculation. This indicates that there is

TABLE 3

Carbon Number Distribution of sn-1,2(2,3)- and X-1,3-diacylglycerols Released from the Triacylglycerols of Abnormal Human Milk and from Horse Milk[a]

Carbon number	Patient (9d5)[b]		Patient (10d)[c]		Horse[b]	
	sn-1,2(2,3)-	X-1,3-	sn-1,2(2,3)-	X-1,3-	sn-1,2(2,3)-	X-1,3-
20					0.9 (0.3)	
22	0.3 (0.4)	0.4 (0.6)	0.4 (0.4)	0.6 (1.5)	2.0 (0.7)	(0.4)
24	3.5 (3.2)	5.2 (4.5)	5.9 (3.7)	6.6 (9.1)	4.0 (2.0)	0.8 (1.4)
26	13.5 (10.1)	15.1 (11.5)	18.0 (13.6)	19.0 (18.5)	7.3 (5.3)	5.2 (5.3)
28	22.0 (20.2)	18.2 (17.0)	24.1 (23.6)	21.5 (19.1)	8.4 (6.3)	10.3 (8.8)
30	18.3 (23.6)	20.8 (25.1)	18.7 (25.2)	20.0 (21.8)	8.0 (9.0)	8.0 (7.6)
32	17.2 (21.0)	16.1 (20.3)	14.4 (16.5)	14.9 (17.9)	13.6 (15.5)	7.1 (8.5)
34	21.4 (17.8)	13.4 (10.9)	14.1 (13.5)	9.9 (6.3)	32.1 (36.6)	21.0 (22.3)
36	3.3 (3.6)	10.2 (10.0)	3.5 (1.4)	6.8 (6.1)	22.7 (23.7)	44.7 (46.0)
38	0.5	0.8	0.7	0.6	1.1	3.0
40			0.2			

[a]Values in brackets have been calculated for a random association of fatty acids in the sn-1-, sn-2- and sn-3-positions from stereospecific analyses (see below). 9d5 and 10d indicate times of milk collection as given in Table 1.

[b]1-random 2-random 3-random.

[c]1,3-random 2-random.

some preferential association of the short and medium chain length fatty acids among themselves in the original triacylglycerol molecules.

A more detailed indication of the molecular association of the fatty acids in the triacylglycerol molecules can be seen from the molecular species distribution obtained for the sn-1,2(2,3)- and the X-1,3-diacylglycerols on polar siloxane columns, which permit a resolution based on both carbon number and degree of unsaturation. Table 4 gives the quantitative results of such analyses for one of the abnormal milk samples and for the sample of the horse milk triacylglycerols. For the patient's milk sample the presence of a higher proportion of unsaturated species in the X-1,3-diacylglycerols indicates that the unsaturated fatty acids are concentrated in the sn-1- and/or sn-3-positions. As shown in Table 3, there are significant differences between the sn-1,2(2,3)- and the X-1,3-diacylglycerols that are derived from the triacylglycerols of horse milk. In contrast to the abnormal human milk there are double maxima in the distribution profile of the X-1,3-diacylglycerols of the horse milk.

Stereospecific Distribution of Fatty Acids

Table 5 gives the distribution of the fatty acids among the sn-1, sn-2- and sn-3-positions of the triacylglycerol molecules of representative samples of the abnormal human and horse milk. The shorter chain fatty acids (C_{10} and C_{12}) are preferentially associated with the sn-3-position, as noted earlier for normal human milk, where they are present in trace amounts only (13), and in horse milk, where they are present in significant amounts (11,18). The saturated C_{14} acid is distributed evenly among the three sn-positions, with the saturated C_{16} acid incorporated largely into the sn-2-position. Stearic acid is preferentially esterified to the sn-1-position, while oleic acid is located mainly in the sn-1- and sn-3-positions of the milk triacylglycerols from both the patient and the normal subject. The small amounts of linoleic acid found in the abnormal human milk sample were distributed among all three positions, as in the normal milk, which contained much more of it. The preferential placement of the medium-chain fatty acids in the abnormal human milk sample is similar to that seen in the horse milk triacylglycerols, although the sn-3-position of the latter contains in addition much linolenic acid. Such a positional distribution of the fatty acids is consistent with the above observed differences in the carbon number distribution of the sn-1,2(2,3)- and of the X-1,3-diacylglycerols derived from the triacylglycerols by Grignard degradation (Tables 3 and 4) and in the fatty acid composition of the corresponding diacylglycerol types (results not shown). It may be noted, however, that a horse milk sample analyzed by Parodi (18) contained more closely similar amounts of C_{12} in the sn-1- and sn-3-positions than the present horse milk sample, with the C_{14} acid being confined largely to the sn-1-position and the sn-2-position.

TABLE 4

Molecular Species of sn-1,2(2,3)- and X-1,3-diacylglycerols Released from Triacylglycerols of Abnormal Human Milk and from Horse Milk by Grignard Degradation[a]

Carbon number: double bond number	Patient (10d)[b,c]		Horse	
	sn-1,2(2,3)-	X-1,3-	sn-1,2(2,3)-	X-1,3-
	Mole %			
20:0			0.9 (0.3)	
22:0	0.4 (0.4)	0.6 (1.5)	1.7 (0.7)	0.4 (0.4)
22:1			0.3	
24:0	5.9 (3.7)	6.6 (9.1)	3.1 (1.8)	0.7 (1.2)
24:1			0.9 (0.3)	0.1 (0.2)
26:0	18.0 (13.6)	19.0 (18.5)	3.7 (2.8)	2.0 (2.1)
26:1			2.8 (1.2)	2.5 (1.8)
26:2			0.9 (0.6)	0.7 (0.7)
28:0	24.1 (26.0)	20.7 (17.9)	3.9 (2.8)	2.4 (1.6)
28:1	(0.3)	0.8 (1.1)	2.8 (3.5)	4.8 (3.6)
28:2		(0.1)	1.7 (0.9)	3.0 (1.7)
30:0	16.1 (21.9)	12.4 (11.7)	3.3 (2.9)	3.7 (1.3)
30:1	1.7 (2.5)	6.3 (9.2)	3.2 (2.9)	2.4 (3.1)
30:2	0.9 (0.8)	1.2 (1.0)	1.5 (1.6)	1.9 (1.6)
32:0	7.4 (8.9)	5.5 (5.8)	4.5 (4.5)	2.0 (0.9)
32:1	5.6 (6.3)	8.2 (10.9)	4.8 (6.0)	2.3 (3.8)
32:2	1.4 (1.4)	1.2 (1.2)	2.1 (2.7)	1.7 (2.0)
32:3			2.2 (2.3)	1.1 (1.7)
34:0	3.1 (3.5)	2.6 (1.6)	(0.6)	(0.1)
34:1	9.0 (8.7)	6.3 (4.1)	13.7 (12.1)	7.2 (5.8)
34:2	1.9 (1.3)	1.0 (0.6)	10.6 (11.2)	7.6 (7.5)
34:3			7.8 (10.8)	6.2 (6.7)
36:0	(0.1)	(0.4)	(0.2)	
36:1	3.5 (0.3)	6.8 (2.1)	2.4 (0.4)	3.0 (0.5)
36:2	(0.5)	(2.9)	3.7 (3.7)	9.4 (7.9)
36:3	(0.4)	(0.6)	6.9 (5.8)	10.7 (11.2)
36:4			7.0 (7.4)	13.0 (14.9)
36:5			2.7 (4.3)	5.5 (7.6)
36:6				3.0 (4.0)

[a]Values in brackets have been calculated for a random association of fatty acids in the sn-1-, sn-2- and sn-3-positions from stereospecific analyses (see below).

[b]10d, milk sample from day 10.

[c]Stereospecific analyses done on sample 9d5.

Composition of Molecular Species

Table 6 gives the carbon number distribution of the sn-2,3-diacylglycerols as determined by direct GLC of the sn-2,3-diacylphosphatidylphenols, along with the carbon number distribution of the sn-1,2-diacylglycerols, as obtained by subtraction of the composition of the sn-2,3-diacylglycerols from that of the sn-1,2(2,3)-diacylglycerols, and of the sn-1,2-, sn-2,3- and the sn-1,2(2,3)-diacylglycerols calculated from the known positional distribution of the fatty acids assuming a 1-random 2-random 3-random distribution. There is fair agreement between the experimental and calculated diacylglycerol profiles, which suggests that the molecular association of the fatty acids in the triacylglycerol molecules is determined largely by their mass proportions. Furthermore, as shown above (see Table 4), the molecular species distribution obtained on the polar columns for the sn-1,2(2,3)- and for the X-1,3-diacylglycerols generated from the triacylglycerols by Grignard degradation, was closely similar to that calculated for these diacylglycerols from the knowledge of the positional distribution of the fatty acids. It was therefore appropriate to calculate the composition of the molecular species of the milk triacylglycerols from the knowledge of the fatty acid composition of the sn-1-, sn-2, and sn-3-positions.

Table 7 compares the calculated and the experimentally determined composition of the carbon numbers of milk fat triacylglycerols of the patient and the horse. The calculation used

TABLE 5

Positional Distribution of Fatty Acids in Milk Triacylglycerols of a Patient with Type I Hyperlipidemia Compared to that of a Normal Subject and of a Horse

Fatty acids	Patient[a]			Normal subject[b]			Horse		
	sn-1-	sn-2-	sn-3-	sn-1-	sn-2-	sn-3-	sn-1-	sn-2-	sn-3-
	Mole %								
8:0							0.2	2.0	3.8
10:0	0.7	1.1	3.7	0.2	0.3	1.4	0.6	1.9	9.2
12:0	10.8	11.1	31.1	1.3	2.7	5.8	2.1	5.8	6.5
14:0	23.9	28.8	28.8	3.2	7.9	7.0	3.3	9.4	5.6
14:1	–	–	–	0.3	0.2	1.5	0.4	0.6	0.0
16:0	22.5	47.7	12.2	16.1	55.8	5.8	17.9	38.4	4.6
16:1	0.8	0.4	0.6	3.6	5.0	7.5	5.1	6.9	5.7
18:0	12.7	2.3	4.7	15.0	3.2	2.0	2.1	1.1	0.1
18:1	25.4	6.5	16.5	46.1	13.4	50.0	32.4	12.0	23.1
18:2	3.3	2.2	3.1	11.0	7.6	14.8	17.6	11.6	20.6
18:3				0.4	0.5	1.6	18.3	10.2	21.6

[a]Sample 9d5, as described in Table 1.

[b]Breckenridge et al. (13).

TABLE 6

Carbon Number Distribution of the sn-1,2-, sn-2,3- and sn-1,2(2,3)-diacylglycerol Moieties of Milk Triacylglycerols of Type 1 Hyperlipidemia Patient as Determined Experimentally and as Calculated from Stereospecific Analyses

Carbon number	Experimental[a]			Calculated[b]		
	sn-1,2-	sn-2,3-	sn-1,2(2,3)-	sn-1,2-	sn-2,3-	sn-1,2(2,3)
	Mole %					
20						
22	0.2	0.40	3.0	0.2	0.7	0.5
24	1.6	5.4	3.5	1.6	4.8	3.2
26	7.7	19.3	13.5	6.3	14.0	10.2
28	16.9	27.1	22.0	15.2	25.2	20.2
30	21.3	17.1	19.2	24.0	23.2	23.6
32	23.4	12.3	17.8	25.7	16.2	21.0
34	25.9	15.1	20.5	22.4	13.1	17.8
36	2.7	2.4	2.6	4.5	2.7	3.6
38	0.3	0.5	0.5			
40	0.1	0.4	0.2			

[a]sn-2,3- and sn-1,2(2,3)-diacylglycerol distributions determined by direct GLC of the corresponding phosphatidylphenols; sn-1,2-diacylglycerol distribution was obtained by subtraction of sn-2,3- from sn-1,2(2,3)-species for sample 9d5.

[b]All diacylglycerol distributions calculated from the stereospecific distribution of the fatty acids assuming non-correlative association.

the 1-random 2-random 3-random method. The experimental data were obtained by multiplication of the fatty acid composition of the sn-2-position by the composition of the molecular species of the X-1,3-diacylglycerols, and by the multiplication of the fatty acid composition of the sn-1-position by the composition of the molecular species of the sn-2,3-diacylglycerols (carbon numbers only). All of these estimates appear to be of about the same order and indicate the general correctness of the overall distribution.

A comparison of the data in Table 7 reveals that there are marked similarities in the association of the medium and long chain fatty acids in the two types of milk fat triacylglycerols. The horse milk fat, however, contains larger proportions of the linolenic acid which, in many instances, would appear to substitute for the saturated medium chain length fatty

TABLE 7

Carbon Number Distribution of Triacylglycerols in Abnormal Human Milk and in Horse Milk as Obtained Experimentally and by Calculation

Carbon number	Patient milk (9d5)					Horse milk			
	Expt.	Calc.[a]	Calc.[b]	Calc.[c]	Calc.[d]	Expt.	Calc.[a]	Calc.[b]	Calc.[c]
	Mole %								
34						0.9	0.3	0.2	0.1
36	1.4	1.1	0.8	0.9	0.8	1.7	0.6	0.5	0.4
38	5.2	3.5	3.0	3.6	3.7	3.0	1.2	1.0	0.7
40	11.0	7.8	7.7	9.1	9.1	4.6	2.1	2.1	1.8
42	14.8	13.5	13.9	15.6	15.1	7.9	4.3	4.8	4.7
44	16.1	18.3	19.0	18.4	19.8	11.3	7.7	8.4	9.0
46	16.3	19.9	21.1	18.3	19.8	10.4	10.0	9.5	10.0
48	12.1	17.2	16.8	15.0	13.8	8.9	11.9	11.2	10.7
50	10.5	11.3	10.4	11.2	9.2	13.4	17.6	17.4	16.6
52	10.0	5.5	6.0	6.4	6.8	23.0	25.4	28.6	27.7
54	2.6	1.7	1.1	1.1	1.0	14.6	18.4	16.1	17.0

[a]1,2,3-random.

[b]1-random 2-random 3-random.

[c]X-1,3-diacylglycerol species X fatty acids in sn-2-position.

[d]sn-2,3-diacylglycerol species X fatty acids in sn-1-position.

acid in the triacylglycerol molecule. As noted from the stereospecific analyses, much of the linolenic acid is associated with the sn-3-position in the horse milk fat triacylglycerols.

Table 8 indicates the composition of the molecular species of triacylglycerols calculated for the patient's milk sample using the 1-random 2-random 3-random method. Again the agreement between the calculated and the determined values appears to be good and suggests that the molecular association of the fatty acids is restricted random despite their highly specific positional placement.

The finding of an apparently non-correlative distribution of the fatty acids among the three positions of the glycerol molecules in the triacylglycerols of the abnormal human milk fat and in the horse milk fat agrees with certain previous findings about the fatty acid distribution in animal milk fat triacylglycerols. Thus, the pig milk triacylglycerols have been demonstrated (19) to give an excellent agreement between the determined triacylglycerol class composition and that calculated by 1-random 2-random 3-random distribution hypothesis, when the triacylglycerols are prefractionated according to degree of unsaturation by argentation TLC and the individual fractions are subjected to stereospecific analysis. Similar results have been obtained for several other mammalian species on the basis of comparisons between the determined carbon number distribution and that calculated from the known positional distribution of fatty acids (18). Furthermore, we have shown elsewhere that the composition of triacylglycerols calculated from the knowledge of the molecular species of the sn-1,2, sn-2,3- and X-1,3-diacylglycerols derived by Grignard degradation gives good agreement with that calculated on the basis of the 1-random 2-random 3-random distribution for peanut oils (20) and for plasma triacylglycerols (21).

Mechanism of Biosynthesis

It appears to have been well established that the triacylglycerols of milk fat are formed exclusively via the phosphatidic acid pathway (2), but some acylation of 2-monoacylglycerols also may take place (22). Table 9 gives the fatty acid composition of the free X-1,2- and X-1,3-diacylglycerols isolated along with the triacylglycerols from the abnormal milk fat as well as of the sn-1,2-diacylglycerol moieties of the milk fat triacylglycerols of the patient, as determined by subtraction of the fatty acid composition of the sn-2,3-diacylphosphatidylphenols from that of the sn-1,2(2,3)-diacylphosphatidylphenols. There is an excellent agreement between the composition of the free X-1,2-diacylglycerols and the sn-1,2-diacylglycerol moieties of the triacylglycerols, which suggests that the free diacylglycerols are of the sn-1,2-enantiomer type and serve as precursors of the sn-1,2-diacylglycerol moieties of the milk fat triacylglycerols. Had the free diacylglycerols originated from an enzymic hydrolysis of the triacylglycerols, they would have possessed

TABLE 8

Composition of Molecular Species of Abnormal Human Milk (9d5) Triacylglycerols[a]

Carbon number[b]	Molecular species[b,c]			Calculated[d]
	Mole %			
38:0	12:0	14:0	12:0	0.97
	14:0	12:0	12:0	0.83
40:0	12:0	14:0	14:0	0.87
	13:0	16:0	12:0	1.61
	14:0	12:0	14:0	0.74
	14:0	14:0	12:0	2.16
42:0	12:0	16:0	14:0	1.44
	14:0	14:0	14:0	1.93
	14:0	16:0	12:0	3.57
	16:0	12:0	14:0	0.70
	16:0	14:0	12:0	2.02
42:1	18:1	12:0	12:0	0.88
44:0	12:0	16:0	16:0	0.63
	14:0	14:0	16:0	0.84
	14:0	16:0	14:0	3.19
	16:0	14:0	14:0	1.81
	16:0	16:0	12:0	3.35
	18:0	14:0	12:0	1.14
44:1	12:0	14:0	18:1	0.51
	18:1	14:0	12:0	2.29
	18:1	12:0	14:0	0.79
46:0	14:0	16:0	16:0	1.40
	16:0	14:0	16:0	0.79
	16:0	16:0	14:0	3.00
	18:0	14:0	14:0	1.02
46.1	12:0	14:0	18:1	0.51
	14:0	14:0	18:1	1.14
	18:1	14:0	14:0	2.05
	18:1	16:0	12:0	3.79
48:0	14:0	16:0	18:0	0.54
	16:0	16:0	16:0	1.31
	18:0	16:0	14:0	1.69
48:1	14:0	16:0	18:1	1.88
	16:0	14:0	16:0	0.89
	18:1	16:0	18:1	3.39
	16:0	14:0	18:1	1.07
48:2	18:1	18:1	12:0	0.51
50:0	16:0	16:0	18:0	0.51
	18:0	16:0	16:0	0.74
50:1	16:0	16:0	18:1	1.76
	18:0	14:0	18:1	0.60
	18:1	16:0	16:0	1.48
50:2	18:1	14:0	18:1	1.21
52:1	18:0	16:0	18:1	1.00
	18:1	16:0	18:0	0.57
52:2	18:1	16:0	18:1	2.00
54:3	18:1	18:1	18:1	0.50

[a]Molecular species making up more than 0.5%; sample 9d5, as described in Table 1.

[b]Carbon number: number of total double bonds.

[c]Fatty acids are given for positions sn-1, sn-2 and sn-3 from left to right.

[d]1-random 2-random 3-random.

fatty acid compositions similar to those of the sn-1,2(2,3)-diacylglycerols of the milk fat triacylglycerols, and would have represented both sn-1,2- and sn-2,3-diacylglycerols in racemic proportions. In fact, there could have been an excess of the sn-2,3-diacylglycerols, since the milk lipase preferentially attacks the sn-1-position of triacylglycerols (23,24). The free X-1,3-diacylglycerols appear to be isomerization products of the sn-1,2-diacylglycerols because of the great similarity in their fatty acid composition.

Since the milk glycerophospholipids also appear to originate predominantly, if not exclusively, from de novo synthesis within the mammary tissue (25), it is conceivable that the sn-1,2-diacylglycerol moieties of the triacylglycerols and those of the glycerophospholipids (largely phosphatidylcholine), would be similar. Table 9 shows that this is not the case. In addition to the differences in the chain length and the degree of unsaturation of the fatty acids of the triacylglycerols and the phosphatidylcholines of the milk fat, there are differences in the molecular association of the fatty acids (results not shown). Apparently, a significant degree of selectivity of diacylglycerol species took place at the time of introduction of the phosphorylcholine moiety (26). These differences in the structure of the diacylglycerol moieties therefore would exclude or minimize the participation of the phosphatidylcholines as intermediates in triacylglycerol formation in the mammary gland (27).

The close agreement between the experimental and the 1-random 2-random 3-random distribution of fatty acids in the milk fat triacylglycerols is consistent with the non-correlative acylation of triacylglycerols first claimed for rat liver (28). However, biochemical evidence to support a 1-random 2-random 3-random distribution in milk triacylglycerols is scanty. It has been shown (29) that the chain length specificity of the acyltransferases is unaffected by the nature of the fatty acid at the sn-1-position of the 1-acyl-sn-glycerol-3-phosphate in the cow. In the lactating rat mammary gland, the acyl CoA specificity was minimally affected by the type of sn-1,2-diacylglycerol acceptor available (30). The best acceptor was sn-1,2-dimyristoyl-, sn-1,2-dipalmitoyl- and sn-1,2-distearoylglycerol in decreasing order of activity. This order of reactivity, however, coincides with the decreasing order of solubility of these diacylglycerols in the aqueous reaction medium. Studies with various preparations of mammary tissue from the cow (29,31) and the rat (30,32) have demonstrated that the sn-glycerol-3-phosphate acyltransferase and the 1-acyl-sn-glycerol-

TABLE 9

Fatty Acid Composition of the Free Diacylglycerols and of the sn-1,2-, sn-2,3- and sn-1,2(2,3)-diacylglycerol Moieties of Triacylglycerols of the Milk (10d) from a Patient with Type 1 Hyperlipidemia

Fatty acids	Free diacylglycerols		Bound diacylglycerols				
	X-1,2-	X-1,3-	sn-1- + sn-2-[a] / 2	sn-2- + sn-3-[a] / 2	sn-1,2(2,3)-[b]	X-1,3-[c]	PC[d]
	Moles %						
10:0	1.9	4.3	0.5	2.5	1.4	2.7	–
12:0	5.2	4.6	12.3	24.7	18.6	27.1	–
14:0	27.4	19.9	32.5	33.0	32.9	32.9	16.7
16:0	43.8	40.8	35.3	28.6	32.0	12.4	35.1
16:1		0.5	0.5	0.3	0.4	0.4	–
18:0	10.7	15.0	5.7	2.0	3.8	6.5	23.5
18:1	8.2	12.3	11.2	7.3	9.2	16.4	10.2
18:2	2.7	2.9	2.3	2.0	2.1	1.8	6.1
Other	–	–	–	–	–	–	8.0

[a]The fatty acid compositions of positions sn-1 and sn-3 for this purpose were estimated by dividing the total composition of positions 1 and 3 according to the proportions found by direct measurement for sample 9d5. The composition of position 2 was determined directly. Samples 9d5 and 10d, as given in Table 1.

[b]As determined for the total sn-1,2(2,3)-diacylphosphatidylphenols.

[c]As determined for the X-1,3-diacylglycerols generated during Grignard degradation.

[d]Phosphatidylcholine.

3-phosphate acyltransferase show very marked specificity for long chain acyl CoAs, although these activities may differ among different cows (31). The composition of the sn-3-position is not determined to any great extent by the specificity of the bovine mammary diacylglycerol acyltransferase (33). The sn-3-position appears to be subject to esterification by any remaining fatty acyl CoAs, giving rise to a highly asymmetric distribution of fatty acids, which is unrelated to the fatty acid composition of any specific position of the glycerol molecule. This non-correlative distribution of acyl groups observed for other milk fats (18,19) appears to hold also for the abnormal milk fat triacylglycerols of the Type 1 hyperlipidemia patient.

Since the completion of this study, altered lipid composition in milk from a patient with Type I hyperlipoproteinemia also has been reported by Berger et al. (34).

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REFERENCES

1. Steiner, G.; Kuksis, A., and Myher, J.J. (1975) Clin. Res. 23, 639A.
2. Smith, S. and Abraham, S. (1975) Adv. Lipid Res. 13, 195-299.
3. Pitas, R.D.; Sampugna, J., and Jensen, R.G. (1967) J. Dairy Sci. 50, 1332-1336.
4. Kuksis, A. (1967) in Lipid Chromatographic Analysis, vol. 1 (G.V. Marinetti, ed.), Marcel Dekker, New York, NY, pp. 239-337.
5. Marai, L.; Breckenridge, W.C., and Kuksis, A. (1969) Lipids 4, 562-570.
6. Folch, J.; Lees, M., and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497-509.
7. Myher, J.J. and Kuksis, A. (1975) J. Chromatogr. Sci. 13, 138-145.
8. Yurkowski, M. and Brockerhoff, H. (1966) Biochim. Biophys. Acta 125, 55-59.
9. Brockerhoff, H. (1967) J. Lipid Res. 8, 167-169.
10. Kuksis, A.; Marai, L., and Myher, J.J. (1973) J. Am. Oil Chem. Soc. 50, 193-201.
11. Breckenridge, W.C. and Kuksis, A. (1967) J. Lipid Res. 8, 473-478.
12. Steiner, G.; Myher, J.J., and Kuksis, A. (1984) Am. J. Clin. Nutr. In Press.
13. Breckenridge, W.C.; Marai, L., and Kuksis, A. (1969) Can. J. Biochem. 47, 761-769.
14. Insull, W., Jr.; Hirsch, J.; James, A.T., and Ahrens, E.H., Jr. (1959) J. Clin. Invest. 38, 443-450.
15. Read, W.W.C.; Lutz, P.G., and Tashjuan, A. (1963) Am. J. Clin. Nutr. 17, 180-183.
16. Harzer, G.; Haug, M.; Dieterich, I., and Gentner, P.R. (1983) Am. J. Clin. Nutr. 37, 612-621.
17. Volpe, J.J. and Vagelos, P.R. (1973) Ann. Rev. Biochem. 42, 21-60.
18. Parodi, P.W. (1982) Lipids 17, 437-442.
19. Christie, W.W. and Moore, J.H. (1970) Biochim. Biophys. Acta 210, 45-56.
20. Manganaro, F.; Myher, J.J.; Kuksis, A., and Kritchevsky, D. (1981) Lipids 16, 508-517.
21. Myher, J.J.; Kuksis, A.; Breckenridge, W.C., and Little, J.A. (1984) Lipids. In Press.
22. Christie, W.W. (1974) Lipids 9, 876-882.
23. Morley, N. and Kuksis, A. (1972) J. Biol. Chem. 247, 6389-6393.

24. Paltauf, F.; Esfandi, F., and Holasek, A. (1974) FEBS Lett. 40, 119-123.
25. Patton, S. (1973) J. Am. Oil Chem. Soc. 50, 178-185.
26. Rao, G.A. and Abraham, S. (1983) Lipids 18, 335-338.
27. Patton, S.; Mumma, R.O., and McCarthy, R.D. (1966) J. Dairy Sci. 49, 937.
28. Slakey, P.M. and Lands, W.E.M. (1968) Lipids 3, 30-36.
29. Marshall, M.O. and Knudsen, J. (1977) Biochim. Biophys. Acta 489, 236-241.
30. Lin, C.Y.; Smith, S., and Abraham, S. (1976) J. Lipid Res. 17, 647-656.
31. Cooper, S.M. and Grigor, M.R. (1980) Biochem. J. 187, 289-295.
32. Kinsella, J.E. (1976) Lipids 11, 680-684.
33. Marshall, M.O. and Knudsen, J. (1979) Eur. J. Biochem. 94, 93-98.
34. Berger, G.M.B.; Spark, A.; Baillie, P.M.; Huskisson, J.; Stockwell, G., and van de Merve, E. (1983) Pediatr. Res. 17, 835-839.

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Studies of Triacyglycerol Structure of Very Low Density Lipoproteins of Normolipemic Subjects and Patients With Type III and Type IV Hyperlipoproteinemia

J.J. MYHER, A. KUKSIS,* W.C. BRECKENRIDGE and **J.A. LITTLE,** *Banting and Best Department of Medical Research and Department of Medicine, University of Toronto, Toronto, Canada M5G 1L6*

ABSTRACT

The triacylglycerols of very low density lipoproteins (VLDL-TG) were analyzed in samples from normal subjects and patients with Frederickson's Type III and Type IV hyperlipoproteinemia. VLDL were obtained by conventional ultracentrifugation, and the triacylglycerols were isolated by thin-layer chromatography (TLC). Representative sn-1,2(2,3)- and sn-1,3-diacylglycerols were generated by Grignard degradation of the triacylglycerols, and were resolved by TLC on borate-treated silica gel. The molecular association of the fatty acids in the diacylglycerol moieties was determined by gas-liquid chromatography with mass spectrometry (GC/MS) of the tertiary-butyldimethylsilyl ethers. The positional distribution of the fatty acids was established by the Brockerhoff stereospecific analysis. The results showed a marked asymmetry in the distribution of the fatty acids in all samples, with the saturated acids predominantly in the sn-1-position and the unsaturated fatty acids distributed about equally between the sn-2- and sn-3-positions. In all instances, the molecular species composition of the sn-1,2-, sn-2,3- and sn-1,3-diacylglycerols was found to be similar to that calculated for 1-random 2-random 3-random distribution of triacylglycerols. There were marked differences in the quantitative composition of the molecular species of the VLDL-TG between normal subjects and patients, but these discrepancies were attributed to differences in the fatty acid composition of the samples.
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INTRODUCTION

Despite considerable experimental attention the causes of hypertriglyceridemia remain obscure. A recent workshop summary of the subject area (1) points to the overproduction of the very low density lipoprotein triacylglycerols (VLDL-TG) as the major defect, but the possibility that this may be associated with decreased clearance of the hepatic VLDL also must be considered. In most studies the VLDL-TG has been treated as a single and uniform moiety of the lipoprotein and no distinction has been attempted between differences in the chemical composition of the VLDL-TG from normal subjects and patients with hyperlipoproteinemia. Limited comparative studies of the composition (2-6) and stereospecific distribution (3-5) of the fatty acids in the VLDL-TG have shown significant differences between patients with hypertriglyceridemia and normolipemic subjects. Thus, Type II patients and normal subjects have been shown to differ in the symmetry of distribution of linoleic acid in the sn-1- and sn-3-positions of the VLDL and LDL (5), while structural analyses of plasma total triacylglycerols have shown statistically significant differences between normal subjects and Type IV patients for palmitic and linoleic acids at the sn-2-, and for oleic and linoleic acids at the sn-3-position (3,4). There have been no confirmatory reports of these findings, and the significance of these differences has remained unexplained in regards to the increased synthesis and/or delayed clearance of the VLDL-TG in hypertriglyceridemia.

We have determined the stereospecific distribution of the fatty acids along with the composition of the molecular species of the VLDL-TG from normal subjects and from patients with Type III and Type IV hyperlipoproteinemia. The results confirm the high asymmetry of the VLDL-TG in both normal subjects and in patients with hypertriglyceridemia, but fail to reveal significant structural differences between normal subjects and patients after correction for discrepancies in the total fatty acid composition of the samples.

MATERIALS AND METHODS

Standards

Purified synthetic sn-1,2, sn-2,3- and sn-1,3-diacylglycerols containing the common fatty acids were available from a previous study (7). Reference fatty acid methyl esters, synthetic triacylglycerols and glycerophospholipids were obtained from Supelco, Inc., Bellefonte, Pennsylvania.

*To whom correspondence should be addressed.

Subjects and Samples

The plasma samples were obtained from three healthy volunteers and from three Type III and three Type IV patients at the St. Michael's Hospital Lipid Clinic, Toronto, Ontario. The subjects lived at home, subsisted on their usual diets and were not taking lipid-lowering drugs. The blood was drawn in the fasting state (12 hrs). The hyperlipoproteinemia condition in each patient was established on the basis of clinical history and the biochemical criteria suggested by the Lipid Research Clinics Program (8). Table 1 gives the age, sex and the plasma total cholesterol and triacylglycerol levels of all subjects. The total lipid profiles of the VLDL fractions are given in Figure 1.

Preparations of Lipoproteins

The VLDL fraction ($d < 1.006$) was isolated essentially according to Hatch and Lees (9), as described in detail previously (10). The identity of the lipoprotein was independently established by double immunodiffusion against rabbit anti-human albumin, anti-human LDL and HDL, as elsewhere described (10). The protein concentration in the lipoprotein fractions was determined by the method of Lowry et al. (11) using bovine serum albumin as standard. The preparation of VLDL was extracted with diethyl ether after the color development. For lipid analyses the VLDL fractions were pooled separately for the patients and for the normal females, while the VLDL of the normal male was analyzed separately.

Analyses of Lipids

Total lipid extracts of the VLDL were obtained by a modified method of Folch et al. (12). The triacylglycerols were isolated by preparative TLC on silica gel plates with petroleum ether (b.p. 30-60 C)-diethyl ether 150-20 as the developing solvent. About 15 mg of triacylglycerol were obtained. Stereospecific analyses of the total triacylglycerols were performed according to Brockerhoff (13). Mixed sn-1,2(2,3)- and sn-1,3-diacylglycerols were generated by Grignard degradation as described by Yurkowski and Brockerhoff (14), but the preparation was scaled down to smaller quantities of triacylglycerols (7). The sn-1,2(2,3)- and the sn-1,3-diacylglycerols were resolved by TLC using borate-impregnated silica gel G and chloroform-acetone 96:4 as the developing solvent (15). The mixed sn-1,2(2,3)-diacylglycerols were converted to the phenylphosphatides and purified by TLC as described by Brockerhoff (16). The phenylphosphatides were exhaustively digested with phospholipase A_2 and the resulting fatty acid, lysophosphatidylphenols and residual phosphatidylphenols were isolated by TLC and their fatty acid composition determined by GLC. The fatty acid composition of the 2-monoacylglycerols was independently obtained by hydrolysis with pancreatic lipase (17) using a commercially available enzyme kit containing diethyl ether-extracted porcine pancreatic lipase, tris(hydrox-

TABLE 1

Plasma Lipids and Clinical Characteristics of Normal Subjects and Patients

Subjects[a]	Plasma total lipids: Cholesterol (mg/dl)	Plasma total lipids: Triglycerides (mg/dl)	Disease
Female	202	110	None
Female	163	112	None
Male	180	131	None
Male	374	438	Type III
Male	232	270	Type III
Male	208	266	Type III
Male	340	1005	Type IV
Male	282	583	Type IV
Male	316	915	Type IV

[a]The subjects were 30-59 years old. The VLDL-TG were pooled separately from the three Type III, three Type IV patients and from the two female control subjects, while the sample from the male control subject was analyzed as such.

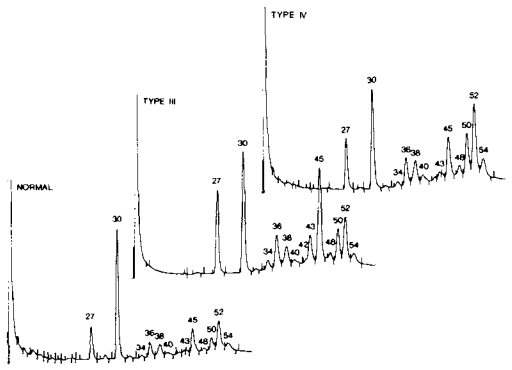

FIG. 1. Total lipid profiles of normolipemic (Normal) and hyperlipemic (Type III and Type IV) plasma as obtained by high temperature gas-liquid chromatography (GLC). Peak 27, trimethylsilyl ether of cholesterol; Peak 30, tridecanoylglycerol internal standard; Peak 34, trimethylsilyl ether of palmitoylsphingosine; Peaks 36-40, trimethylsilyl ethers of diacylglycerols of a total number of 34 to 38 acyl carbons; Peaks 43-45, cholesteryl esters of fatty acids with a total number of 16-18 acyl carbons; Peaks 48-54, triacylglycerols with a total number of 48-54 acyl carbons. Sample size 1 μl of an approximately 1% solution in silylation mixture. Attentuation: 100 times full sensitivity. Other operating conditions and instrumentation as given in text.

methyl)-aminomethane buffer and gum Arabic (Analabs, North Haven, Connecticut). The monoacylglycerols were isolated by TLC on borate impregnatted silica gel with chloroform-acetone 88:12 as the developing solvent (15).

Intact triacylglycerols and diacylglycerol t-BDMS ethers were resolved according to molecular weight by GLC on 3% OV-1 (7,18). This method was also satisfactory for pyrolysis GLC of the sn-2,3-diacylphosphatidylphenols. Fatty acid methyl esters were analyzed on columns prepared with 10% EGSS-X and 3% SILAR 5CP as described (19). The fatty acid methyl esters were prepared using 6% sulfuric acid in absolute methanol, or with IN sodium methoxide in methanol-benzene 60:40. The molecular species of the various diacylglycerol preparations were determined by GC/MS of their tertiary-butyldimethylsilyl (t-BDMS) ethers as previously described (20,21). The t-BDMS ethers were prepared by reacting the diacylglycerols with tertiary-butyldimethylchlorosilane-imidazole reagent (Applied Science Laboratories, State College, Pennsylvania) at 80 C for 20 min (20). The t-BDMS ethers of diacylglycerols were purified by TLC using toluene-diethyl ether 97:3 as the developing solvent. Total lipid profiles of the VLDL were determined as described (10).

Calculations

The positional distribution of the fatty acids in the triacylglycerols was calculated according to the methods outlined by Brockerhoff (13). The sn-2-position was obtained from the fatty acid composition of the sn-2-monoacylglycerols released by pancreatic lipase. The fatty acid composition of the sn-3-position of the triacylglycerol molecule was calculated from the formula:

$$\text{sn-3-} = (\text{FA of TG} \times 3) - (\text{FA of MG} + \text{FA of Lyso PL})$$

The monoacylglycerols and lysophosphatides are representative, respectively, of the sn-2- and sn-1-positions. Theoretical compositions of triacylglycerols and diacylglycerols were calculated on the basis of 1-random 2-random 3-random distribution (7), which assumes that the fatty acid composition of each position of the acylglycerol molecule is independent of the composition of the other two positions. The corresponding carbon number and double bond number profiles of the triacylglycerols, sn-1,2(2,3)- and sn-1,3-diacylglycerols were obtained by summing and normalizing the appropriate products of multiplication.

An index of non-randomness of distribution (INR) of molecular species of diacylglycerols was computed by the formula

$$\text{INR} = \Sigma\,(\text{EXPT} - \text{CALC})^2$$

where EXPT and CALC are the experimental and calculated compositions, and the summation is made over the entire series of molecular species. A value close to zero indicates a complete randomness, while duplicate analyses of a mixture of natural sn-1,2- or sn-2,3-diacylglycerols give INR values of about 20. However, in the present study INR values up to twice the experimental error were considered insignificant.

RESULTS

Analysis of Total Triacylglycerols

The composition and positional distribution of fatty acids in the VLDL triacylglycerols of the Type III and Type IV patients and of the normolipemic subjects of comparable age are given in Table 2. The results for the controls

TABLE 2

Positional Distribution of Fatty Acids in VLDL Triacylglycerols from Normal Subjects and Patients with Type III and Type IV Hyperlipoproteinemia

Fatty acids	Normals				Type III				Type IV			
	Pos. 1[a]	Pos. 2	Pos. 3	Total	Pos. 1	Pos. 2	Pos. 3	Total	Pos. 1	Pos. 2	Pos. 3	Total
	Mole %											
14:0	3.3	2.1	0.9	2.1	1.2	4.0	2.2	2.5	3.1	3.5	0.3	2.3
16:0	51.3	7.8	8.1	22.4	66.9	14.6	8.1	29.9	66.3	17.8	6.5	30.2
16:1	6.7	8.0	2.9	5.9	5.3	15.8	6.6	9.2	4.3	8.0	3.2	5.2
18:0	4.4	0.6	2.8	2.6	5.1	1.9	3.0	8.3	6.4	2.2	3.8	4.1
18:1	22.5	43.1	56.3	40.6	18.2	49.1	59.7	42.3	14.6	45.6	62.6	40.9
18:2	10.9	37.6	26.5	25.0	3.3	14.6	17.9	11.9	3.9	22.3	21.3	15.2
18:3	0.9	0.8	2.5	1.4	–	–	2.6	0.9	1.3	0.6	2.0	1.3

[a]Positions 1, 2 and 3 represent sn-1, sn-2 and sn-3-positions in sn-glycerol of the triacyl-sn-glycerols.

agree well with previous analyses of the positional distribution of fatty acids in total plasma triacylglycerols of normolipemic subjects (3). The sample is highly asymmetric with respect to all the major fatty acids. Thus, 76% of all palmitic acid occurs in the sn-1-position, while the sn-3-position contains 12% of it, with the rest being found in the sn-2-position. In contrast, 46% of oleic acid occurs in the sn-3-position, while the sn-1-position contains only 18% of it. Likewise, the sn-3-position contains about 2.5 times as much linoleic acid as the sn-1-position. A comparable asymmetric distribution was found for these fatty acids in the VLDL triacylglycerols of the Type III and Type IV patients. There was a highly selective placement of the palmitate in the sn-1-position of the triacylglycerols, while the oleate and linoleate were preferentially associated with the sn-3-position. A similar trend of distribution of these fatty acids for the Type IV subjects has been reported by Parijs et al. (4), who analyzed total plasma triacylglycerols. In the control subjects the sn-2-position showed a markedly higher content of linoleic acid than the primary positions, while in the patients both sn-2- and sn-3-positions contained about the same proportions of the acid. There were marked differences in the total fatty acid composition of the VLDL triacylglycerols among the subjects, which were presumably due to dietary differences.

Figure 2 gives the carbon number profiles of the VLDL triacylglycerols of the normal subjects and of the patients with Type III and Type IV hyperlipoproteinemia. The quantitative estimates of the triacylglycerol carbon numbers are compared in Table 3. It is seen that all subjects possess closely similar carbon number proportions. They all contain the bulk of the species in the form of C_{50}-C_{52} triacylglycerols, along with smaller amounts of C_{46} species. It may be noted that the experimental carbon number distribution approximates closely that obtained by 1-random 2-random 3-random calculation based on the fatty acid composition in Table 2.

Analysis of Derived Diacylglycerols

Figure 3 gives the carbon number distributions of the sn-1,2(2,3)- and the sn-1,3-diacylglycerols derived from the VLDL-TG of the normal subjects and the patients with Type III and Type IV hyperlipoproteinemia. A quantitative evaluation of the peak areas of the diacylglycerols is given in Table 4. It is seen that in all instances the diacylglycerols contain the same carbon numbers (Peaks 34 and 36). However, while the sn-1,2(2,3)-diacylglycerols contain these peaks in about equal proportions, the sn-1,3-diacylglycerols are characterized by a 2-3-fold greater excess of Peak 34 over Peak 36.

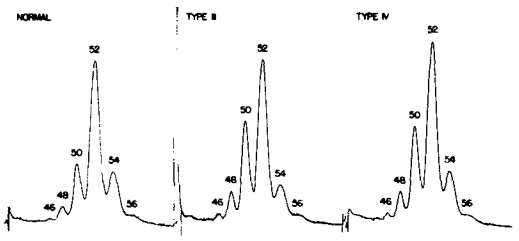

FIG. 2. Carbon number profile of triacylglycerols of VLDL from a normolipemic (Normal) and hyperlipemic (Type III and Type IV) subjects. Peaks 45-56 represent triacylglycerols with a total number of 45-56 acyl carbons. GLC conditions as previously described (7).

TABLE 3

Distribution of Carbon Numbers of Triacylglycerols in VLDL-TG from Normal Subjects and Patients with Type III and Type IV Hyperlipoproteinemia

Carbon number	Normals		Type III		Type IV	
	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.
46	0.5	0.4	1.1	1.0	1.0	0.5
48	3.4	3.4	6.1	7.4	5.8	5.2
50	16.7	17.9	25.6	28.3	22.7	24.7
52	56.7	50.9	51.2	48.7	51.0	52.6
54	19.4	28.0[a]	13.1	14.5	16.0	16.6
56	3.4	–	2.9	–	3.5	–

[a]The single normolipemic male subject analyzed separately gave a complete agreement between the observed and calculated carbon number of the VLDL-TG.

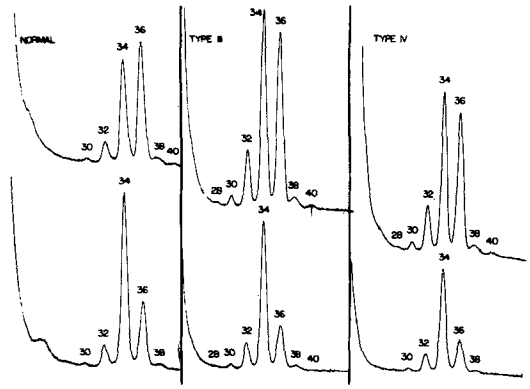

FIG. 3. Carbon number profiles of sn-1, 2(2,3)- and sn-1,3-diacylglycerols derived by Grignard degradation of VLDL-TG of normal and hyperlipemic subjects. Peaks 30-40, trimethysilyl ethers of diacylglycerols with a total of 30-40 acyl carbons: Upper profiles, sn-1,2(2,3)-diacylglycerols; lower profiles, sn-1,3-diacylglycerols. GLC conditions as in Figure 2.

TABLE 4

Distribution of Carbon Numbers in Diacylglycerol Moieties of VLDL-TG from Normal Subjects and Patients with Type III and Type IV Hyperlipoproteinemia

Carbon number	Normals		Type III		Type IV	
	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.
	Mole %					
sn-1,2(2,3)						
30	1.3	1.1	1.8	2.2	1.7	1.9
32	8.6	8.5	13.4	16.1	11.8	14.3
34	41.0	38.4	45.1	44.1	43.7	43.4
36	49.2	52.3	37.8	36.0	42.5	40.9
sn-1,3-						
30	0.8	0.9	1.6	0.8	1.9	0.5
32	8.0	9.6	12.2	12.2	11.7	9.5
34	63.3	55.2	64.1	63.8	64.1	65.9
36	27.9	33.5	22.0	22.5	22.1	23.7

Peaks 30-36 normalized to 100% for purposes of comparing observed and calculated values.

TABLE 5

Distribution of Molecular Species in sn-1,2(2,3)-Diacylglycerol Moieties of VLDL-TG from Normal Subjects and Patients with Type III and Type IV Hyperlipoproteinemia

Molecular species[a]	Normals		Type III		Type IV	
	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.
	Moles %					
30:0	0.6	0.8	0.9	1.7	1.1	1.6
30:1	0.7	0.3	0.0	0.5	0.6	0.3
32:0	2.2	2.4	4.4	5.7	5.6	6.7
32:1	4.5	4.5	8.0	8.8	4.9	5.7
32:2	1.9	1.6	1.0	1.6	1.3	1.9
34:0	0.7	0.5	0.1	1.4	1.1	1.7
34:1	18.1	16.3	28.9	24.9	26.8	24.0
34:2	19.2	17.9	17.0	16.1	14.0	15.2
34:3	3.0	3.7	1.4	2.7	1.8	2.4
36:0	–	0.0	–	0.1	0.3	0.1
36:1	1.3	1.8	2.1	2.7	2.9	3.2
36:2	16.6	18.5	20.2	19.9	20.2	19.0
36:3	21.4	22.9	12.8	10.9	14.4	14.4
36:4	8.3	8.3	2.7	2.2	3.1	3.8
36:5	1.6	0.8	–	0.2	1.6	0.4
Σ(Obs.-Calc.)^2[b]	4		31		39	

[a]Molecular species identified by total number of acyl carbons: total number of double bonds; positional isomers and enantiomers are not distinguished.

[b]Index on non-randomness.

This is due to the preferential location of the palmitic acid in the sn-1-position and its association with C_{18} fatty acids in the sn-3-position. The above carbon number distributions were closely similar to those calculated by the 1-random 2-random 3-random distribution using the data from the positional analysis of fatty acids and summing the multiplication products by carbon number. Furthermore, these carbon number distributions agreed closely with those calculated from the data of Gordon et al. (5) and Parijs et al. (4), attesting to the general asymmetry of the structure of the parent triacylglycerols.

The molecular association of fatty acids in the triacylglycerols was estimated by chromatographic and mass spectrometric examination of the sn-1,2(2,3)- and the sn-1,3-diacylglycerols derived from the VLDL-TG by random degradation with the Grignard reagent. The composition of the molecular species of the sn-1,2(2,3)-diacylglycerol moieties of the triacylglycerols of the normal subjects and the patients is given in Table 5. The theoretical values were derived by the 1-random 2-random 3-random calculation using the positional distribution of fatty acids given in Table 2. There is a rather close agreement between the experimental and calculated compositions of the molecular species for both normal subjects and patients. All patients possess, however, a higher proportion of the monoenes and dienes (34:1 and 36:2) than the normolipemic subjects, which contain relatively more of the trienes and tetraenes (36:3 and 36:4). On the basis of these similarities between the experimental and calculated values it may be concluded that the fatty acids in each of three positions of the triacylglycerol molecule are esterified independently of the two other positions.

The composition of the molecular species of the sn-1,3-diacylglycerol moieties of the VLDL-TG is given in Table 6. In this instance the correspondence between the experimental and the calculated values is not as good as that seen for the sn-1,2(2,3)-diacylglycerol moieties. This is due to the inherent difficulty of preparing the sn-1,3-diacylglycerols without contamination from the isomerization products of the sn-1,2(2,3)-diacylglycerols noted previously (13). Nevertheless, there are noteworthy differences seen between the molecular species of the sn-1,3-diacylglycerols in the normal subjects and patients. Thus, the sn-1,3-diacylglycerols from the VLDL-TG of the patients are higher in the monoenes (34:1) and lower in the dienes and trienes (34:2 and 36:3) than those of the normal subjects, which is in accordance with the findings for the sn-1,2(2,3)-diacylglycerols.

Figure 4 compares the carbon number distribution in the sn-2,3-diacylglycerol moieties of the sn-2,3-diacylphosphatidylphenols, which represent the sn-2,3-diacylglycerol moieties of

TABLE 6

Distribution of Molecular Species in sn-1,3-Diacylglycerol Moieties of VLDL-TG from Normal Subjects and Patients with Type III and Type IV Hyperlipoproteinemia

Molecular species[a]	Normals		Type III		Type IV	
	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.
			Moles %			
30:0	–	0.7	1.2	0.6	1.5	0.4
30:1	0.8	0.2	0.4	0.2	0.4	0.1
32:0	1.5	4.3	5.8	5.6	5.2	4.4
32:1	4.9	4.1	6.5	6.0	5.5	4.4
32:2	1.6	1.2	–	0.6	1.0	0.7
34:0	0.6	1.8	3.3	2.4	1.6	3.0
34:1	37.0	31.0	47.7	41.9	47.0	42.8
34:2	21.6	18.9	13.1	16.6	13.8	17.6
34:3	4.1	3.5	–	2.9	1.7	2.5
36:0	0.1	0.1	–	0.5	–	0.3
36:1	4.0	3.1	3.5	3.6	4.9	4.6
36:2	11.1	14.1	12.2	11.9	10.8	10.7
36:3	9.1	12.2	4.1	5.4	5.0	5.7
36:4	3.6	4.0	2.2	1.1	1.4	2.0
36:5	–	–	–	–	–	0.4
Σ(Obs.-Calc.)^2[b]	4		31		39	

[a]Molecular species identified by number of acyl carbons: total number of double bonds; positional isomers and enantiomers are not distinguished.

[b]Index of non-randomness.

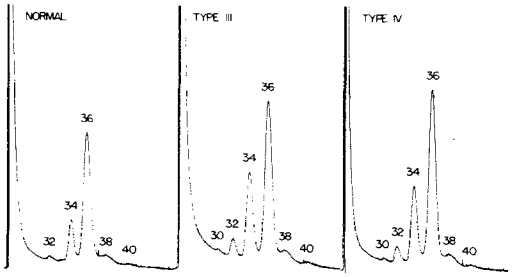

FIG. 4. Carbon number profiles of sn-2,3-diacylglycerols derived by pyrolysis of sn-2,3-diacylphosphatidylphenols prepared from VLDL-TG by Grignard degradation, synthesis of phosphatidylphenols and stereospecific hydrolysis with phospholipase A_2. Peaks 32-40, diacylglycerols with a total number of 32-40 acyl carbons. GLC conditions as in Figure 2.

the original VLDL-TG from the normal subjects and from the patients with Type III and Type IV hyperlipoproteinemia. Table 7 gives the determined composition of the sn-2,3-diacylglycerols by carbon number in comparison to the corresponding compositions calculated from random distribution.

It is seen that the Type III patients possess a somewhat higher proportion of the C_{32} and C_{34} diacylglycerols than the Type IV patients or the control subjects, but this discrepancy is probably due to the slightly higher proportion of palmitic acid in the sn-2- and sn-3-positions of the original VLDL-TG as seen from Table 2. There were no apparent differences between the determined and calculated distributions of the carbon numbers, which attests further to the apparent independence of the molecular association of the fatty acids among the different positions of the glycerol molecule.

TABLE 7

Distribution of Carbon Numbers in Diacylglycerol Moieties of VLDL-TG from Normal Subjects and Patients with Type III and Type IV Hyperlipoproteinemia

Carbon number	Normals		Type III		Type IV	
	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.
			Moles %			
sn-1,2(2,3)						
30	1.3	1.1	1.8	2.2	1.7	1.9
32	8.6	8.5	13.4	16.1	11.8	14.3
34	41.0	38.4	45.1	44.1	43.7	43.4
36	49.2	52.4	37.8	36.0	42.5	40.9
sn-2,3[a]						
30	–	0.4	–	1.3	–	0.4
32	2.3	4.3	5.6	9.2	5.0	3.0
34	18.2	23.0	30.7	35.1	27.4	31.0
36	79.5	72.4	62.6	54.5	67.6	65.6

[a]sn-2,3-diacylphosphatidylphenols.

Reconstitution of Molecular Species of Triacylglycerols

Since the experimental and calculated distributions for the sn-1,2(2,3)-, sn-2,3- and sn-1,3-diacylglycerols agreed closely when compared on the basis of the proportions of the various unsaturation classes within each carbon number, it was concluded that the 1-random 2-random 3-random calculation also would give a reasonable approximation of the molecular species composition of the triacylglycerols. This assumption is experimentally verified in Table 3, which compares the experimental and calculated carbon number distributions of the triacylglycerols. The agreement is excellent. Table 8 gives the calculated compositions of the major molecular species of the VLDL-TG of control subjects and of Type III and Type IV patients based on the 1-random 2-random 3-random distribution. The molecular species are arranged in order of increasing double bond number within each carbon number. Table 8 includes a total of 45 species, which account for about 80% of the total VLDL-TG. Among

TABLE 8

Distribution of Molecular Species of Triacylglycerols in VLDL of Normal Subjects and Patients with Type III and Type IV Hyperlipoproteinemia

Carbon number	Molcular species			Normals	Type III	Type IV
					Moles %	
48:0	16:0	16:0	16:0		0.791	0.767
48:1	16:0	14:0	18:1	0.606	1.597	1.452
48:1	16:0	16:0	16:1		0.644	0.644
48:1	16:0	16:1	16:0		0.856	
50:1	16:0	16:0	18:1	2.252	5.831	7.387
50:1	16:0	18:1	16:0	1.790	2.660	1.965
50:2	14:0	18:1	18:1	0.800		0.884
50:2	16:0	16:0	18:2	1.060	1.748	2.513
50:2	16:0	16:1	18:1	2.310	6.310	3.320
50:2	16:0	18:1	16:1	0.641	2.167	0.967
50:2	16:0	18:2	16:0	1.562	0.791	0.961
50:3	14:0	18:2	18:1	0.698		
50:3	16:0	16:1	18:2	1.087	1.892	1.129
50:3	16:0	18:2	16:1	0.559	0.644	
52:1	16:0	18:1	18:0	0.619	0.985	1.148
52:1	16:0	18:0	18:1		0.758	0.913
52:1	18:0	16:0	18:1			0.713
52:2	16:0	18:0	18:1	12.448	19.610	18.925
52:2	16:0	18:2	18:0	0.540		0.561
52:2	18:1	16:0	18:1	0.988	1.586	1.586
52:2	18:1	18:1	16:0	0.785	0.723	
52:3	16:0	18:1	18:2	5.859	5.879	6.439
52:3	16:0	18:2	18:1	10.859	5.831	9.255
52:3	16:1	18:1	18:1	1.625	1.553	1.227
52:3	18:1	16:1	18:1	1.013	1.716	0.731
52:3	18:1	18:2	16:0	0.685		
52:3	18:1	18:1	16:1		0.589	
52:3	18:1	16:0	18:2			0.553
52:4	16:0	18:1	18:3	0.552	0.854	0.604
52:4	16:0	18:2	18:2	5.111	1.748	3.149
52:4	16:1	18:1	18:2	0.765		
52:4	16:1	18:2	18:1	1.418		0.600
52:4	18:1	16:1	18:2		0.514	
52:5	16:1	18:2	18:2	0.667		
54:2	18:0	18:1	18:1	1.067	1.494	1.826
54:3	18:0	18:1	18:2	0.502		0.621
54:3	18:0	18:2	18:1	0.931		0.893
54:3	18:1	18:1	18:1	5.459	5.334	4.167
54:4	18:1	18:1	18:2	2.569	1.599	1.418
54:4	18:1	18:2	18:1	4.762	1.586	2.038
54:4	18:2	18:1	18:1	2.644	0.967	1.113
54:5	18:1	18:2	18:2	2.241		0.693
54:5	18:2	18:1	18:2	1.244		
54:5	18:2	18:2	18:1	2.307		0.544
54:6	18:2	18:2	18:2	1.086		

Positions of enantiomeric triacylglycerols read from left to right as follows: sn-1-, sn-2- and sn-3- position.

the individual molecular species there are marked quantitative differences between the normal subjects and patients, which are due to differences in the fatty acid composition of the samples, but not to their arrangement in the triacylglycerol molecules. Thus, a close inspection of Table 8 reveals that the hypertriglyceridemic subjects contain relatively more of triacylglycerols with sn-1,2-dipalmitoyl moieties and less of the triacylglycerols with the sn-1,2-dilinoleoylglycerol moieties. The high proportion of the triacylglycerols with sn-1,2-

dipalmitoylglycerol moieties is due in part to the greater proportion of palmitic acid in these samples and in part to the specific increase of this acid in the sn-2-position with increasing concentration of the total palmitate. The opposite changes apparently take place for the linoleic acid. All samples appear to contain similar proportions of the triacylglycerols with sn-1,2-dioleoylglycerol moieties. The changes in the palmitic and linoleic acid content of the sn-positions of the VLDL-TG correspond to those reported by Parijs et al. (4) for the total triacylglycerols of Type IV patients. In Type III patients there was a significant increase in the palmitoleic acid content of sn-2-position of the VLDL-TG, when compared to normal subjects. There have been no previous stereospecific analyses of the total or VLDL-TG of Type III patients.

DISCUSSION

Validity of Calculation of Molecular Species of Triacylglycerols

The calculation of the molecular species of triacylglycerols on the basis of the 1-random 2-random 3-random distribution is justified by the experimental demonstration of the existence of non-correlative distribution of fatty acids in the sn-1,2(2,3)- and sn-1,3-diacylglycerols. A further experimental validation of the calculated distribution is provided by the close agreement between the calculated and the determined carbon numbers for the sn-2,3-diacylglycerols. The 1-random 2-random 3-random distribution is consistent with certain metabolic facts about triacylglycerol biosynthesis and secretion by animal and human liver. Thus, it is known that acyltransferases exist which are specific for the esterification of sn-1- and sn-2-positions of sn-glycerol-3-phosphate leading to the formation of phosphatidic acids with markedly different complements of fatty acids in the sn-1- and sn-2-positions (22,23). It also is well known that the sn-3-position of the triacylglycerol molecule is synthesized last and that a fatty acid pool different from that utilized in the synthesis of the phosphatidic acids is utilized (24). There also may be differences in the subcellular sites involved in the synthesis of the different acylglycerol derivatives (25). A non-correlative esterification of fatty acids in the various glycerol positions previously has been claimed by Slakey and Lands (26) for total rat liver triacylglycerols, but Christee and Moore (27) could not confirm it for plasma triacylglycerols of the pig.

Triacylglycerol Differences Among Normal Subjects and Patients

Estimates of relative indices of non-randomness of fatty acid distribution by subtracting the experimental from the calculated distribution of molecular species in the sn-1,2(2,3)- and sn-1,3-diacylglycerols and in the carbon number distributions of triacylglycerols gave differences of the order of the experimental error of analysis. Apparently, both normal subjects and patients biosynthesized VLDL-TG with the same degree of positional specificity, and of the same degree of non-correlative molecular association. Likewise, there were no obvious differences in the relative composition of the VLDL-TG between the Type III and Type IV hyperlipoproteinemia patients. Both types were characterized by the presence of triacylglycerols of comparable asymmetry and apparently represented the products of biosynthesis via the phosphatidic acid pathway of the liver. This observation is consistent with the current belief (28) that the elevated VLDL-TG in hypertriglyceridemia is due to an overproduction of VLDL rather than to its delayed clearance. If the clearance had been delayed, it is possible that differences might have been detected between normal subjects and patients due to the specificity of lipoprotein lipase, which might have attacked certain species more readily than others (29). However, in Type III hyperlipoproteinemia Fainaru et al. (30) have observed a heterogeneous mix of lipoproteins in the $d < 1.006$ plasma fraction, which could have included chylomicron remnants. As a result, the latter triacylglycerols should have possessed more symmetrical distributions for the saturated and unsaturated fatty acids because the chylomicron triacylglycerols are formed largely via the monoacylglycerol pathway, which is less stereospecific than the phosphatidic acid pathway (31,32). Since all the triacylglycerol mixtures in this study were markedly asymmetrical, it must be concluded that the chylomicron remnants contributed little to the VLDL-TG of plasma. Previous work has shown (29) that lipoprotein lipase of normal subjects hydrolyzes all of the common triacylglycerol species at about the same rate by attacking the sn-1-position in preference to the sn-3-position. It is not known what the specificity is for the lipoprotein lipase of patients with Type III and Type IV hyperlipoproteinemia. In conclusion the present detailed comparisons of the VLDL-TG composition of control subjects and patients with Type III and Type IV hyperlipoproteinemia fail to reveal any structural abnormalities.

It would be desirable to confirm this identity of VLDL-TG structures in normal subjects and patients with hyperlipoproteinemia by analyzing individual samples from a larger number of subjects subsisting on controlled diets.

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REFERENCES

1. Lippel, K.; Tyroler, H.; Eder, H.; Gotto, A. Jr., and Vahouny, G. (1981). Arteriosclerosis 1, 406-417.
2. Allard, C.; Davignon, J.; Marcel, Y.L.; Goulet, C.; Kuba, K.; Alteresco, M.; Caradec, Y., and Plante, D. (1973). Can. J. Biochem. 51, 1509-1514.
3. Parijs, J.; De Weerdt, G.A.; Beke, R., and Barbier, F. (1974). Lipids 9, 937-938.
4. Parijs, J.; De Weerdt, G.A.; Beke, R., and Barbier, F. (1976). Clin. Chim. Acta 66, 43-52.
5. Gordon, D.T.; Pitas, R.E., and Jensen, R.G. (1975). Lipids 10, 270-283.
6. Jensen, R.G.; Clark, R.M.; Gerrior, S.A.; Fey, M.B., and Gotto, A.M. Jr. (1979). Lipids 14, 691-694.
7. Myher, J.J.; Marai, L.; Kuksis, A., and Kritchevsky, D. (1978). Lipids 12, 775-785.
8. Lipid Research Clinics Program. Manual of Laboratory Operations. Vol. 1: Lipid and Lipoprotein Analysis. DHEW Publication Number (NIH) 75-628. Washington, DC: National Institutes of Health, 1974.
9. Hatch, F.T. and Lees, R.S. (1968). Advan. Lipid Res. 6, 1-68.
10. Kuksis, A.; Myher, J.J.; Geher, K.; Hoffman, A.G.D., Breckenridge, W.C.; Jones, G.J.L., and Little, J.A. (1978). J. Chromatogr. 146, 393-412.
11. Lowry, O.H.; Rosebrough, N.J.; Farr, A.K., and Randall, R.J. (1951). J. Biol. Chem. 193, 265-275 (1951).
12. Folch, J.; Lees, M., and Sloane-Stanley, G.H. (1957). J. Biol. Chem. 26, 497-509.
13. Brockerhoff, H. (1971). Lipids 6, 942-956.
14. Yurkowski, M. and Brockerhoff, H. Biochim. Biophys. Acta 125, 55-59.
15. Thomas, A.E.; Scharoun, J.E. III, and Ralston, H. (1965). J. Am. Oil Chem. Soc. 42, 789-792.
16. Brockerhoff, H. (1965). J. Lipid Res. 6, 10-15.
17. Mattson, F.H. and Volpenhein, R. (1961). J. Lipid Res. 2, 58-62.
18. Kuksis, A.; Marai, L., and Myher, J.J. (1973). J. Am. Oil Chem. Soc. 50, 193-201.
19. Myher, J.J.; Marai, L., and Kuksis, A. (1974). Anal. Biochem. 62, 188-203.
20. Myher, J.J.; Kuksis, A.; Marai, L., and Yeung, S.K.F. (1978). Anal. Chem. 50, 557-561.
21. Kuksis, A.; Breckenridge, W.C.; Myher, J.J., and Kakis, G. (1978). Can. J. Biochem. 56, 630-639.
22. Possmayer, F.; Scherphof, G.L.; Dubbelman, T.N.A.R., and Van Deenen, L.L.M. (1969). Biochim. Biophys. Acta 176, 95-110 (1969).
23. Yamashita, S.; Hosaka, K., and Numa, S. (1973). Eur. J. Biochem. 38, 25-31.
24. Lands, W.E.M.; Pieringer, R.A.; Slakey, P.M., and Zschocke, A. (1966) Lipids 1, 444-448.
25. Higgins, J.A. and Barnett, R.J. (1971) J. Cell Biol. 50, 102-120.
26. Slakey, P.M. and Lands, W.E. (1968). Lipids 3, 30-36.
27. Christee, W.W. and Moore, J.H. (1970). Biochim. Biophys. Acta 210, 46-56.
28. Bell, U.; Grundy, S.M.; Crouse, J.R., and Zech, L. (1982). Arteriosclerosis 2, 44-57.
29. Morley, N.H.; Kuksis, A., and Buchnea, D. (1974). Lipids 9, 481-487.
30. Fainaru, M.; Mahley, R.W., Hamilton, R.L., and Innerarity, T.L. (1982). J. Lipid Res. 23, 702-714 (1982).
31. Breckenridge, W.C.; Yeung, S.K.F.; Kuksis, A.; Myher, J.J., and Chan, M. (1976). Can. J. Biochem. 54, 137-144.
32. Bugaut, M.; Myher, J.J.; Kuksis, A., and Hoffman, A.G.D. (1984) Biochim. Biophys. Acta 792, 254-269.

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METHODS

Purification of Lysosomal Cholesteryl Ester Hydrolase From Rat Liver by Preparative Isoelectric Focusing

RABBE KLEMETS[a,*] and BO LUNDBERG,[b] [a]*Wallac Biochemical Laboratory, P.O. Box 10, SF - 20101 Turku 10, Finland and* [b]*Department of Biochemistry and Pharmacy, Åbo Akademi, Porthansgatan 3, SF - 20500 Åbo 50, Finland*

ABSTRACT

Ion-exchange chromatography and preparative isoelectric focusing (PIEF) were compared to produce a stable rat liver lysosomal cholesteryl ester hydrolase of high specific activity. The PIEF purification method proved to be more rapid and easier to perform. PIEF purification involved the following steps: i) osmotic shock of the lysosome fraction, ii) $(NH_4)_2$ SO_4 precipitation (10-70%, w/v), iii) Sepharose CL-6B gel filtration, and iv) PIEF. The enzyme was purified 60-120-fold with a yield of 2-4%. The activity of the purified enzyme was best restored by stabilizing with a 0.5% (w/v) albumin solution. The purified enzyme produced one major band on SDS-polyacrylamide gel electrophoresis having a MW of 58,500 daltons. Gel filtration showed a MW of 58,000 daltons. The optimum pH of the enzyme was 4.5, and the isoelectric point was 6.0-6.2. The specific activity of hydrolysis of cholesteryl oleate and triolein increased by similar rates during purification.
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INTRODUCTION

The enzymic activity of acid cholesteryl ester hydrolase (ACEH) (E.C. 3.1.1.13) is restricted mainly to the lysosomes of liver and other tissues (1,2,3). ACEH purified from liver has been reported to have hydrolytic activity also towards triacylglycerols (4). A change in or a deficiency of lysosomal cholesteryl ester hydrolase activity leads to different metabolic disorders such as Wolmans disease (5) and cholesteryl ester storage disease (6). It also has been regarded as a factor responsible for the accumulation of cholesteryl esters in smooth muscle cells of atherosclerotic arteries (7,8).

Although ACEH from rat liver has been purified by different methods, the enzyme preparations have been unstable and the procedures difficult to reproduce (9,10,11). The yield of pure enzyme also has been quite small. The mechanism for the action of cholesteryl esterase on cholesteryl esters embedded in supersubstrates with different physicochemical properties is not yet fully understood. Nor is it clear by what mechanism the different lipid components in the supersubstrate affect the enzymatic activity. In a previous study, we investigated the effect of substrate properties on ACEH using a partial purified enzyme preparation (12). The aim of the present study was to develop a rapid and efficient method of producing a stable and pure ACEH suitable for studies on substrate specificity and enzyme kinetics.

*To whom correspondence should be addressed.

MATERIALS AND METHODS

Chemicals

Cholesteryl [1-^{14}C] oleate, specific activity 34 mCi/mmol, and glycerol tri [1-^{14}C] oleate, specific activity 30-60 mCi/mmol, were obtained from The Radiochemical Centre, Amersham, United Kingdom. Glycerol tri [1-^{14}C] oleate was purified by Florisil column chromatography before use, and the radiochemical purity was 99% as tested by thin layer chromatography (TLC) on silica gel plates. Cholesteryl oleate was purchased from Merck, Darmstadt, Federal Republic of Germany. It was found to be chromatographically pure by TLC and was therefore used without further purification. Glycerol trioleate, sodium taurocholate and p-nitrophenylacetate were obtained from Sigma Chemicals, St. Louis, Missouri. Egg phosphatidylcholine was isolated in our laboratory according to the method described by Lundberg (13). Glycerol trioleate was purified by Florisil column chromatography before use. Acrylamide, N,N'-methylenebisacrylamide and N,N,N',N'-tetramethylethylenediamine were obtained from Eastman Kodak Co., Rochester, New York. Triton X-100 was obtained from BDH Chemicals, Poole, United Kingdom. Sephadex and Sepharose chromatographic media, as well as the calibration proteins, were obtained from Pharmacia, Uppsala, Sweden. Ultrodex, Ampholines and Ampholine PAG-Plates, pH 3.5 −9.5, were obtained from LKB Produkter, Bromma, Sweden.

Preparation of the Lysosome Fraction

The livers of 4-6 Sprague-Dawley rats (each weighing 150-250 g) were used for each isolation. The subcellular fractionation procedures of the lysosomal fraction were the same as described earlier (12) except that homogenization was performed in 8 volumes of 0.5 M sucrose, 1 mM EDTA, in 0.01 M Tris-HCl (pH 7.5). The lysosomal pellet from the 12,000 × g (20 min) centrifugation step was frozen to −20 C and then thawed by adding 50 ml of the cold Tris buffer containing 1 mM EDTA per rat liver. This solution was frozen and thawed 3 times and is hereafter called the osmotically shocked lysosome fraction. This fraction was stored at −20 C.

The release of ACEH from lysosome membranes was tested by using different concentrations of Triton X-100 and sodium chloride. These releasing agents were added to samples of the osmotically shocked lysosome fraction and the mixtures stirred for 2 hr at 4 C. One set of test tubes was used for Triton X-100 and a different set for NaCl. The final concentration of Triton X-100 in the samples was adjusted to 0, 0.2, 0.5 and 1.0% (w/v) respectively in the Tris buffer (pH 7.4). The final concentration of NaCl in the samples was adjusted to 0.1 and 0.3 M, respectively. After completion of the release procedure the samples were centrifuged at 50,000 × g for 30 minutes at 4 C. The pellet and supernatant were separated and protein content and ACEH activity were determined in each fraction. Protein was determined according to Lowry et al. (14).

Purification Procedure

All the purification steps were performed at 0-4 C. The osmotically shocked lysosome fraction was diluted, if necessary, with 0.01 M Tris-HCl (pH 7.5), to produce a protein concentration of 5 mg/ml or less. This protein solution was subjected to 10-70% (w/v) $(NH_4)_2SO_4$ precipitation. The pellet was collected and dissolved in the Tris buffer containing 0.25 M sucrose. The solution was applied onto a Sepharose CL-6B column (2.5 × 50 cm), which was eluted with 0.05 M Tris-HCl buffer (pH 7.0) at a flow rate of 60 ml/hr. The elution profile was monitored at A_{280}. Fractions showing ACEH activity were pooled and concentrated with an Amicon ultrafiltration cell using a PM-30 filter membrane (Amicon Corporation, Lexington, Massachusetts). The concentrate was washed with 2 volumes of the Tris buffer containing 0.25 M sucrose in the ultrafiltration cell. This fraction was further purified by PIEF using a granulated Ultrodex gel.

The gel was prepared by suspending 4 g Ultrodex in 100 ml of distilled, deionized water containing 4.7 ml Ampholine, pH 5-7, and 0.4 ml Ampholine, pH 3.5-10. The gel was spread on a 2 mm-thick glass plate (12 × 20 cm) to a thickness of 3-4 mm and a hair dryer used to reduce the water content by about 39%. A strip of gel (1 cm wide) was then carefully cut and removed from the plate and mixed with the ACEH concentrate in a beaker before being poured back into the 1 cm wide trough from which it came. The anode solution used was 1% H_3PO_4 and the cathode solution 1% NaOH. The glass plate was put on a Multiphor cooling chamber maintained at 0 C. A LKB 2103 Constant Power Supply unit was used to supply a constant power of 40 W. After the focusing had finished (about 4 hours), the pH gradient was measured with a surface electrode. Gel pieces (1 × 2 cm) were then scraped off the plate and eluted in a LKB 2117-502 PEGG elution column with 3 ml of the Tris buffer containing 0.25 M sucrose. The eluate was immediately assayed for ACEH activity and the protein concentration was determined using a modified Lowry assay (15).

In order to compare the PIEF method with conventional purification methods, the enzyme concentrate obtained after Amicon ultrafiltration was separately purified by chromatography using DEAE-Sephadex A-50 and CM-Sephadex C-50. The DEAE-Sephadex purification procedure was performed according to (16) with the following modifications. The buffer used was 0.05 M Tris-HCl (pH 7.8) and the linear gradient ranged from 0-0.4 M NaCl. The CM-Sephadex purification procedure was performed according to (9) at pH 5.6. After the enzyme activity was measured, the active fractions were pooled and dialysed with the ultrafiltration cell using 0.01 M Tris-HCl (pH 7.5), 0.25 M sucrose, and stored at −20 C.

Cholesteryl Ester Hydrolase Assay

The vesicle substrate used for the determination of ACEH contained 20 mg egg phosphatidylcholine and 500 μg cholesteryl [1-^{14}C] oleate per ml. The substrate was prepared in 0.1 M NaCl, 0.01 M Tris-HCl buffer (pH 7.4), and 0.02% NaN_3 as described earlier (12). Each assay contained 100 μl substrate preparation, 150 μl 0.15 M acetate buffer, pH 5.0, and 25-100 μl of the enzyme preparation. The final incubation volume was made up to 400 μl with distilled water. The incubation was carried out for 15 min at 37 C on a rotary shaker table immersed in a water bath. The labelled free fatty acid formed was measured according to Pittman et al. (17). The radioactivity was calcu-

lated using a Triton-toluene scintillation cocktail, which contained per liter 667 ml toluene, 333 ml Triton X-100, 6 g PPO and 0.8 g dimetyl-POPOP. All measurements were made in duplicate.

Triacylglycerol Hydrolase Assay

Substrate preparation for the Triacylglycerol hydrolase assay was prepared in the same manner as that for the ACEH assay and contained 20 mg egg phosphatidylcholine and 4 mg glycerol tri [1-^{14}C] oleate per ml. This gave a substrate preparation that contains mainly lecithin and triolein emulsion particles (18). The enzyme assay was similar to the one described for ACEH except that 50 μl of the substrate preparation was used.

Non-specific Esterase Assay

Non-specific carboxylesterase activity was assayed using p-nitro-phenylacetate as the substrate. A solution of p-NPA (4 mM) was prepared by adding 2 ml, 100 mM p-NPA dissolved in methanol, to 48 ml of a mixture containing 0.15 M acetate buffer, pH 5.0, and 0.4% Triton X-100 (w/v). The mixture was stirred constantly during the addition procedure. The enzyme assay was initiated by adding 50 μl of enzyme preparation to 4 ml of the substrate solution. The incubation was carried out for 15 min at 37 C. The release of p-nitrophenol was recorded at 400 nm.

Characterization of the Purified Enzyme

The degree of purification was routinely followed by conventional polyacrylamide gel electrophoresis. Duplicate samples were run; one gel slab was stained to give a visual estimate of protein content and the other was stained in order to compare the accordance with non-specific activity. The latter test was carried out by incubating the gel at room temperature in the following substrate solution: 2 mM p-NPA in 0.15 M acetate buffer, 0.4% Triton X-100, and 2% methanol. Analytical isoelectric focusing of the purified enzyme was performed using Ampholine PAG-Plates, pH 3.5-9.5.

The molecular weight of the purified enzyme was determined by SDS-polyacrylamide gel electrophoresis according to the method of Weber and Osborn (19). Purified fractions of PIEF were dialysed against 0.01 M Tris-HCl (pH 7.4), 0.25 M sucrose on Amicon ultrafiltration cell using a PM-10 filter before being applied to the gel rod. Electrophoresis calibration proteins obtained from Pharmacia were used as standards. The molecular weight also was determined by gel filtration chromatography using a Sephadex G-150 column (2.5 × 50 cm). The column was eluted with 50 mM Tris-HCl (pH 7.0). Gel filtration calibration proteins obtained from Pharmacia were used to calibrate the column.

The stability of the PIEF purified, and gel filtrated enzyme was tested by storage at −20 C in 0.01 M Tris-HCl (pH 7.4) containing, respectively, one of the following stabilizers: 0.25 M sucrose, 20% (w/v) glycerol and 0.5% (w/v) bovine albumin. The stability of the osmotically shocked lysosome fraction, ammonium sulphate precipitated enzyme and the enzyme purified by ion exchange chromatography were

TABLE 1

Release of Cholesteryl Ester Hydrolase From Rat Liver Lysosomes

Release procedure tested	Total protein (mg)	Total activity (nmol × h^{-1})	Total activity (% of control)
1. Lysosomal fraction (= control)	143	39194	100
2. Lysosomal pellet dissolved in 0.01M Tris-HCl, pH 7.5, 1 mM EDTA	143	55264	141
3. No. 2, 3 times frozen and thawed	143	68590	175
4. Lysosomal fraction homogenized in an all glass apparatus	117	40083	102

Release procedure tested, lysosomal fraction dissolved in 1) 0.25 M sucrose, 0.01 M Tris-HCl, pH 7.5, 1 mM EDTA, 2) dissolved in 0.01 M Tris-HCl, pH 7.5, 1 mM EDTA, 3) numbr 2 frozen and thawed as a thin layer (0.5 cm thick) 3 times, and 4) number 1 homogenized in all-glass apparatus. The vesicle substrate, containing 20 mg egg, phosphatidylcholine and 500 μg cholesteryl [1-^{14}C] oleate per ml was used in the assays.

tested with 0.25 M sucrose in 0.01 M Tris-HCl (pH 7.5).

ACEH purified by PIEF was characterized for its optimum pH, initial reaction velocity and maximum reaction rate (Vmax) using the vesicle substrate.

RESULTS

Release of ACEH

Table 1 shows the results of experiments designed to release the maximum amount of cholesteryl ester hydrolase from lysosomes but without simultaneously inhibiting the enzyme activity, as is generally the case when using detergents, such as Triton X-100, as a releasing agent. When the lysosomal pellet was dissolved in 0.01 M Tris-HCl (pH 7.5), 1 mM EDTA, the total yield increased by 41% as compared to the same buffer containing sucrose. Three freezing and thawing sequences increased the total enzyme activity still further. Increasing the freezing and thawing cycles from three to ten times did not help the yield.

Osmotic shock followed by freezing and thawing alone without releasing agents resulted in about 90% of the enzyme activity being found in the supernatant fraction. Triton X-100 used at a concentration of 0.2% (w/v) clearly affected the protein distribution between the supernatant and precipitate fractions but had a very strong inhibitory effect on ACEH activity. A more pronounced effect was obtained with the higher Triton X-100 concentrations. When NaCl was used, no solubilizing effect was noticed but there was slight inhibition of total ACEH activity.

Based on the results from Table 1, fraction No. 3 was used for further purification.

Purification

Cholesteryl ester hydrolase was treated with a wide range (10-70%) of ammonium sulfate saturation to achieve a good yield. This step usually yielded a twofold increase in ACEH activity. The ammonium sulphate precipitate was further purified by gelfiltration on Sepharose CL-6B (data not shown). The ACEH eluted as a broad double peak immediately after the second major protein peak. This type of elution pattern was observed during several elutions. Acid trioleylglycerol hydrolase activity and part of the p-NPA hydrolase activity coincided with cholesteryl oleate hydrolysis. When using Sephadex G-200 for gel filtration, one sharp peak for cholesteryl oleate hydrolysis was obtained. This activity coincided with the protein peak. Prolonged concentration of the enzyme after gel filtration with an Amicon ultrafiltration cell using a PM 30 filter inactivated the enzyme.

Figure 1 shows the ACEH activity, p-NPA hydrolysis and protein pattern found in a typical isoelectric focusing experiment. The ACEH activity coincided with p-NPA hydrolysis activity. Specific activity for cholesteryl oleate hydrolysis from six similar purifications varied within a range of 2369-3050 nmol × mg^{-1} ×

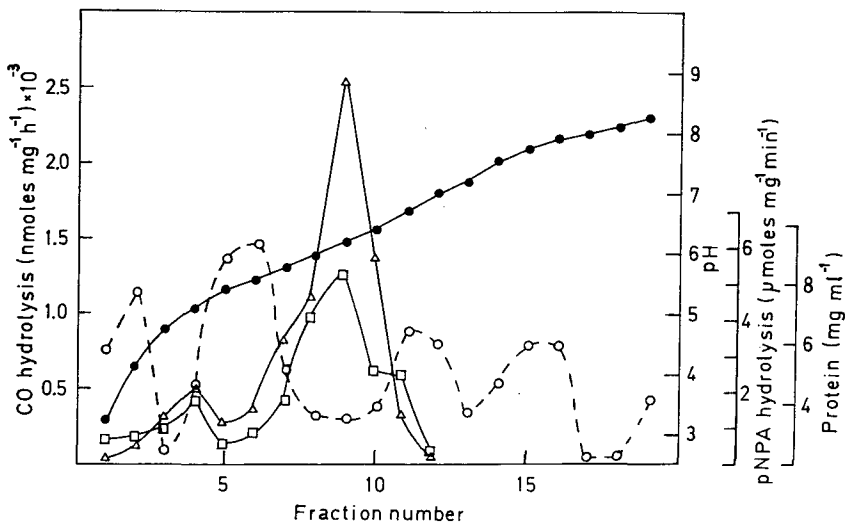

FIG. 1. Purification of cholesteryl ester hydrolase on PIEF using a pH gradient (•) of pH 5-8. Details of the procedure and the assays are described in the text. Cholesteryl oleate (△) and pNPA (□) hydrolysis. Protein concentration (○).

TABLE 2

Purification of Cholesteryl Ester Hydrolase From Rat Liver Lysosomes

No.		Total protein (mg)	Specific activity			CO-hydrolysis		Ratio		Purification factor CO-hydrolysis
			CO-hydrolysis	p-NPA-hydrolysis	TO-hydrolysis	Total activity	Yield %	$\frac{CO}{p\text{-}NPA}$ ($\times 10^{-3}$)	$\frac{CO}{TO}$	
1	Homogenate	10652	25.7	0.49	63.5	273800	(100)			1
2	Osmotically shocked lysosome fraction	267	385	0.79	320	101200	(37)	8.1	1.2	15
3	$(NH_4)_2 SO_4$ 10-70% w/v	124	731	1.94	841	90300	(33)	6.3	0.9	28.4
4	Sepharose CL-6B	46.1	990	2.13	669	45648	(16.7)	7.7	1.5	38.5
5	Amicon PM 30	32.6	940	N.D.	N.D.	30614	(11.2)	–	–	36.6
6	PIEF	2.4	2567	17.2	1536	6133	(2.2)	2.5	1.7	99.9
7	PIEF of osmotically shocked lysosome fraction	2.8	2880	31.7	N.D.	7940	(2.9)	1.5	–	112
8	CM Sephadex of 5	2.7	2032	N.D.	3153	5476	(2.0)	–	0.64	79.1
9	DEAE Sephadex of 5	1.9	1860	N.D.	N.D.	3560	(1.3)	–	–	72.4

Abbreviations: ND = not determined; CO = cholesteryl oleate; p-NPA = p-nitrophenylacetate; TO = triolein.

Specific activity is expressed as nmol (^{14}C)-oleic acid released per hour per mg protein for CO- and TO-hydrolysis, and as μmol p-nitrophenol released per min per mg protein for p-NPA hydrolysis. Total activity of CO-hydrolysis is expressed as nmol (^{14}C)-oleic acid released per hour. Assays and purification procedures are described under materials and methods.

h^{-1}. When the osmotically shocked lysosome fraction was directly purified by PIEF, the usual result was a very high yield of enzyme activity in a single purification step.

In order to compare the final purification step, ACEH was purified on CM-Sephadex C-50 and DEAE-Sephadex A-50 columns. With the cationic exchanger, the enzyme was eluted as one peak at a concentration of 0.1 M NaCl, and with DEAE-Sephadex at a concentration of approximately 0.17 M NaCl (data not shown).

The results of the purification procedures are shown in Table 2. The degree of purification usually was slightly higher with PIEF than with the ion exchange chromatographic methods. The yield of pure enzyme was similar in all three methods tested except that PIEF produced the enzyme in a more concentrated form.

Characteristics of the Enzyme

The pH optimum of the purified ACEH was 4.5. The enzyme produced an initial velocity lasting 20 min with the vesicular substrate and had a V_{max} value of 2.083 nmol × mg^{-1} × h^{-1}. Its molecular weight was 58,500 daltons as determined by SDS-polyacrylamide electrophoresis and 58,000 daltons as determined by Sephadex G-150 gelfiltration. The isoelectric point measured by analytical isoelectric focusing was 6.0-6.2.

On polyacrylamide gel electrophoresis the PIEF fraction showed one major band at a R_f-value of 0.12-0.14 and a weak band with a slightly higher R_f-value. When polyacrylamide gel slabs were incubated in the 2 mM p-NPA solution, the yellow band showing esterase activity appeared in the gels at the R_f-value of 0.12-0.14.

The stability of ACEH in different media at −20 C is shown in Figure 2. No loss in ACEH activity was observed when the osmotically shocked lysosome fraction (in 0.25 M sucrose, 0.01 M Tris-HCl, pH 7.4) and the dissolved $(NH_4)_2SO_4$ precipitate fraction were stored at −20 C up to four weeks. However, the lysosomal fraction is sensitive to higher temperatures and a decrease of 15% ACEH activity was observed during a 6 hr period of storage at room temperature (20 C). When the enzyme prepared after the gel filtration step was stored in 0.25 M sucrose, 0.01 M Tris-HCl, pH 7.4, about 25% of its activity was lost in 10 days at −20 C. But no loss in activity for 10 days was found when stored in 0.5% BSA. When the purified ACEH was stabilized with bovine serum albumin, only 10% of the original activity was lost after 1 week of storage. The stabilizing effect of the tested compounds: BSA, glycerol and sucrose, each in 0.01 M Tris HCl, pH 7.4, and then the Tris Buffer by itself, decreased in the order in which they are listed here.

Ampholine, pH 5-7, did not inhibit the enzyme at a concentration of 0.2% (w/v) which was the approximate concentration of Ampholine in the fractions used for analysis after PIEF purification. At an assay concentration of 1.25% (w/v) Ampholine caused a decrease of the original activity with 30%.

DISCUSSION

A stable, pure enzyme is needed to study the kinetics and enzyme-substrate interaction of lipolytic enzymes. By using PIEF as the final purification step, a 100 fold purification of lysosomal cholesteryl ester hydrolase is achieved in one day.

The purified enzyme had a molecular weight of approximately 58,000, a pH optimum of 4.5 when using the vesicle substrate and an isoelectric point of 6.2. These values are in agreement with earlier reports (9,20).

ACEH and the acid lipase are obviously partly associated with lysosomal membranes (10,11). Triton X-100 is often used to release these enzymes from membranes even though nonionic detergents have been reported to interfere with cholesteryl ester hydrolysis (9, 12). In our enzyme release experiments, we found no advantage in using Triton X-100. The

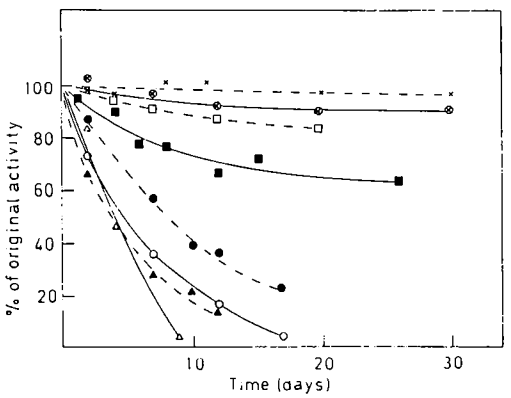

FIG. 2. Stability of purified cholesteryl oleate hydrolase. Stability of osmotically shocked lysosome fraction (X), Sepharose CL-6B (■) and CM-Sephadex C-50 (△) fractions stored in 0.01 M Tris-HCl buffer (pH 7.4), 0.25 M sucrose. Stability of dissolved $(NH_4)_2SO_4$ precipitate (⊗). PIEF purified fraction stored in: a) 0.01 M Tris-HCl buffer (pH 7.4) (▲), b) a + 0.25 M sucrose (○), c) a + 0.5% (w/v) BSA (□) and d) a + 20% (w/v) glycerol (●).

most effective method of releasing ACEH was to osmotically shock the lysosome fraction followed by freezing and thawing.

Specific hydrolytic activity towards cholesteryl oleate and triolein increased throughout the purification procedure although the ratio of cholesteryl oleate hydrolysis to triolein hydrolysis did not vary significantly between the osmotically shocked lysosome fraction and the PIEF-fraction. This may indicate that the same enzyme catalyzes the hydrolysis of both lipids. There are several reports which support this hypothesis in the rat (9), rabbit (16) and human liver (4).

ACEH activity eluted from the Sepharose CL-6B column as a broad double peak. However, on Sephadex G-200 the enzyme eluted as one narrow peak. The reason for this remains unclear but the existence of different sized enzyme-phospholipid complexes could be one reason. Support for this assumption is given by a variety of reported molecular weights for the purified or partially purified enzyme (16), and by the fact that the enzyme is activated by the addition of intact lysosomes (11).

The purification fold with PIEF was clearly higher than with the ion exchange media tested when applying the same sample. The yield of purified enzyme was similar with the methods tested. Similar low yields of acid cholesteryl ester hydrolase have been reported by others (9). In comparison to column chromatographic methods, the advantage of using preparative flat bed isoelectric focusing on a granulated gel as the final purification step is the rapid isolation of the enzyme in a concentrated band (21). The method is suitable for small scale purification even though quite large amounts of protein can be applied to the gel (22). When drying the gel under an air dryer, it is advisable to apply the crude enzyme preparation as a band to avoid inactivation of the enzyme. The purified enzyme usually was collected at pH 5.8-6.2, which correlates well with the reported isoelectric point.

ACEH and acid lipase have been reported to be unstable enzymes when purified from different mammalian liver sources (10,15). In this study it has been clearly shown that it is possible to increase the stability of the purified enzyme at −20 C by the addition of stabilizers. Bovine serum albumin proved to be the most effective stabilizer in maintaining enzyme activity, and about 90% of original activity remained after 12 days of storage at −20 C. Glycerol also had some stabilizing effect but sucrose proved ineffective.

REFERENCES

1. Nilsson, Å.; Norden, H., and Wilhelmsson, L. (1973) Biochim. Biophys. Acta 296, 593-603.
2. Stokke, K.T. (1972) Biochim. Biophys. Acta 280, 329-335.
3. Takano, T.; Black, W.J.; Peters, T.J., and de Duve, C. (1974) J. Biol. Chem. 249, 6732-6737.
4. Warner, T.G.; Dambach, L.M.; Shin, J.H., and O'Brien, J.S. (1981) J. Biol. Chem. 256, 2952-2957.
5. Sloan, H.R. and Fredrickson, D.S. (1972) in The Metabolic Basis of Inherited Disease, 3rd edn. (Staubury, J.B.; Wyngaarden, J.B., and Fredrickson, D.S., eds.), pp. 802-832, McGraw-Hill, New York.
6. Goldstein, J.L.; Dana, S.E.; Faust, J.R.; Beadet, A.L., and Brown, M.S. (1975) J. Biol. Chem. 250, 8487-8495.
7. Brecher, P.; Kessler, M.; Clifford, C., and Chobanian, A. (1973) Biochim. Biophys. Acta 316, 386-394.
8. Kritchevsky, D. and Kothari, H.V. (1973) Biochim. Biophys. Acta 326, 489-491.
9. Brown, W.J. and Sgoutas, D.S. (1980) Biochim. Biophys. Acta 617, 305-317.
10. Teng, M.H. and Kaplan, A. (1974) J. Biol. Chem. 249, 1064-1070.
11. Takano, T. and Imanaka, T. (1978) Acta Histochem. Cytochem. 11, 323-336.
12. Lundberg, B.; Klemets, R., and Lövgren, T. (1979) Biochim. Biophys. Acta 572, 492-501.
13. Lundberg, B. (1973) Acta Chem. Scand. Ser. B. 27, 3545-3549.
14. Lowry, O.H.; Rosebrough, N.J.; Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 263-275.
15. Bensadoun, A. and Weinstein, D. (1976) Analytical Biochemistry 70, 241-250.
16. Imanaka, T.; Muto, K.; Ohkuma, S., and Takano, T. (1981) Biochim. Biophys. Acta 665, 322-330.
17. Pittman, R.C.; Khoo, J., and Steinberg, D. (1975) J. Biol. Chem. 250, 4505-4511.
18. Ekman, S. and Lundberg, B. (1978) Acta Chem. Scand. B 32, 197-202.
19. Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406-4412.
20. Brecher, P.; Chobanian, J.; Small, D.M., and Chobanian, A.V. (1976) J. Lipid Research 17, 239-247.
21. Delincée, H. and Radola, B.J. (1975) Eur. J. Biochem. 52, 321-330.
22. Radola, B.J. (1975) Biochim. Biophys. Acta 386, 181-195.

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COMMUNICATIONS

Dihomo-γ-linolenic Acid Reverses Hypertension Induced in Rats by Diets Rich in Saturated Fat

CEDRIC H. HASSALL* and STEPHEN J. KIRTLAND, *Roche Products Limited, Welwyn Garden City, Hertfordshire, England*

ABSTRACT

This study has shown that hypertension induced in rats by a diet rich in saturated fat (16% coconut oil, 4% palmitic acid by weight) is reversed by the addition of the essential fatty acid, dihomo-γ-linolenic acid (DHLA), at 5.0% but not at 0.5% of dietary energy. This potent effect of DHLA has been attributed to modulation of prostaglandin biosynthesis.
Lipids 19:699-703, 1984.

INTRODUCTION

Diets rich in saturated fat have been shown to elevate blood pressure of man (1) and animals (2,3) and to exacerbate salt-induced (3-6) and spontaneous hypertension (6,7). These hypertensive effects have been ascribed to limitation of the production of prostaglandins from dietary linoleates (1,4-7). The conversion of linoleic acid (18:2ω6) to prostaglandins proceeds in mammals through desaturation and homologation steps to DHLA (20:3ω6) and arachidonic acid (20:4ω6), the immediate precursors of prostaglandin E_1 (PGE_1) and prostaglandin E_2 (PGE_2), respectively (8). This pathway is controlled by genetic, hormonal and nutritional factors, which limit the synthesis of DHLA from dietary linoleates (9-11). It was therefore of interest to investigate the potential of DHLA to modulate hypertension induced with saturated fat in an animal model. Since dietary DHLA is directly available for prostaglandin synthesis (12-14), diets containing this acid could well show effects against such hypertension different from those of diets enriched with linoleates (2-7).

MATERIALS AND METHODS

Animals and Diets

Male, Wistar rats maintained on a certificated, pelleted diet (B.P. Nutrition, Essex, see footnote a, Table 3) were allocated to three groups (n = 12) of similar body weight (approximately 78 g) and blood pressure (approximately 125 mm Hg). Each group was then fed one of three synthetic diets. These diets were isocaloric (4.3 $cal \cdot g^{-1}$) with the same general composition (see Table 1). Fat represented 41.7% of total dietary energy. The larger part of this fat (33.3% total energy) was provided by coconut oil (Loders and Nucoline Ltd., London); the remainder was a mixture of palmitic acid (94% purity by gas liquid chromatography (GLC), British Drug Houses Ltd., Dorset) and chemically-synthesised DHLA (98% purity by GLC, Roche Products Ltd., Herts). The total amount of both acids added was the same for all diets, but their proportions were varied to provide diets with increasing levels of DHLA at 0, 0.5 and 5.0% of dietary energy. All diets contained 0.7% dietary energy as linoleic acid (from coconut oil). Bile salts, a mixture of sodium glycocholate and sodium taurocholate (Oxoid) were added with the prospect that these might improve the absorption of lipids. In preliminary studies, it was noted that the pigmentation of feces was reduced in rats fed a high fat diet, possibly because of their inability to completely absorb the high levels of fat. The introduction of bile salts to the diet did tend to normalise fecal color. However, bile salts were not essential to induce hypertension and had no significant effect on essential fatty acid status.

Diets and drinking water were fed ad libitum and allocated as indicated in the Results section and legend to Figure 1. Food intakes were measured weekly on a cage basis (4 rats).

Blood Pressure Measurements

Systolic blood pressure was determined by tail cuff sphygmomanometry (15) using a W+W Recorder (Kontron Instruments, Herts).

Determination of Prostaglandins in Urine

Rats were housed individually in metabolism cages and urines were collected for 24 hr. PGE's were extracted into acidified ethyl acetate and isolated after separation by argentation thin-layer chromatography as previously

*To whom correspondence should be addressed.

TABLE 1

Compositions (Weight %) of the Experimental Diets

Constituent	Diet		
	Non-supplemented	With DHLA (0.5% energy)	With DHLA (5.0% energy)
Coconut oil[a]	16.0	16.0	16.0
Palmitic acid	4.0	3.76	1.59
DHLA	–	0.24	2.41
Casein, vitamin and fat free[b]	20.0	20.0	20.0
Cellulose (solkafloc)	4.0	4.0	4.0
Sucrose	46.2	46.2	46.2
Vitamin/mineral mix[c]	7.6	7.6	7.6
Methionine	0.2	0.2	0.2
Bile salts	2.0	2.0	2.0

[a]Fatty acid composition (weight %) by GLC: 8:0, 5.0%; 10:0, 4.7%; 12:0, 45.0%; 14:0, 21.0%; 16:0, 10.1%; 18:0, 3.2%; 18:1, 8.4%, and 18:2, 2.2%.

[b]82.0% of protein by weight.

[c]Providing the following, as mg per kg diet: vitamin A, 3.9; vitamin D_3, 0.064; vitamin B_1, B_2 and B_6, 12.8 each; vitamin B_{12} 0.013; vitamin C, 2560; vitamin E, 700; vitamin K, 0.024; folic acid, 2.6; nicotinic acid, 26; pantothenic acid, 24; choline, 1050; biotin, 0.128; inositol, 640; p-aminobenzoic acid, 128; iron, 128; copper, 0.064, and fluorine, 12.8. And, as weight (%) of diet: calcium, 0.89; phosphorus, 0.64; magnesium, 0.06; potassium, 0.64; and sodium, 0.24 (0.15% sodium chloride).

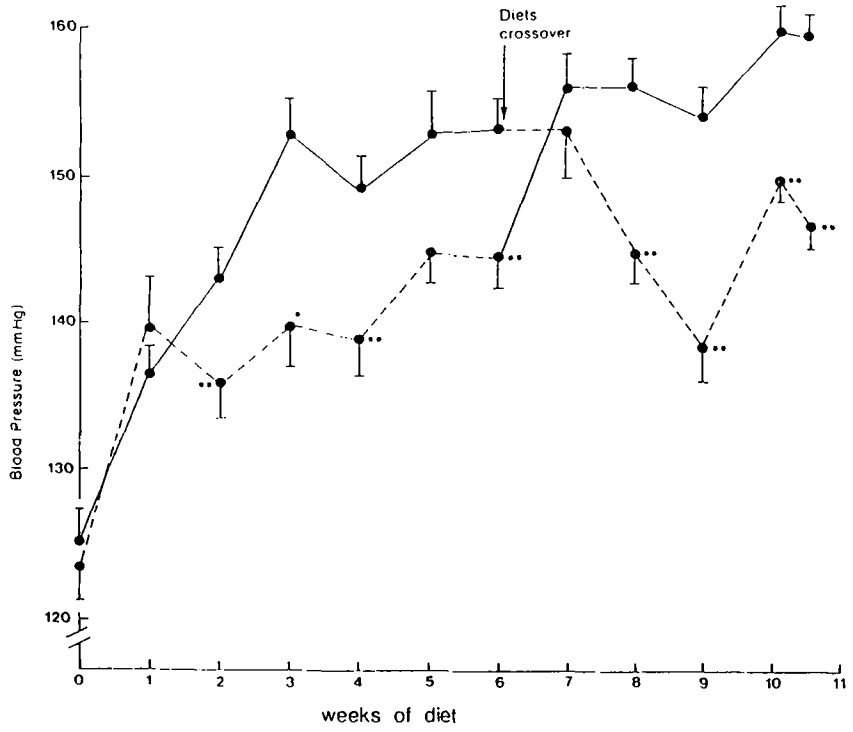

FIG. 1. Influence of diet on blood pressure of the rat. Rats (n=12) were fed for 6 weeks a diet rich in fat (41.7% dietary energy as fat, 36.3% saturated) and low in essential fatty acids (–●–). Over the same period, another group (n=12) were fed a saturated fat-rich diet supplemented with DHLA (31.3% energy saturated fat, 5% energy as DHLA, (---●---). After 6 weeks, diets of the two groups of rats were crossed over. Differences in blood pressure ($\bar{x}$ ± s.e.m.) of rats receiving the different diets are indicated; *, $p<0.05$; **, $p<0.02$.

described (16). Rf's for authentic standards of PGE_1 and PGE_2 were 0.67 and 0.48 respectively. PGE's were determined by radioimmune assay. The cross reactivity of antisera to PGE_1 and PGE_2 (Institut Pasteur Production) is given in Table 2.

Lipid Analysis

Blood was obtained by aortic puncture under Sagatal anesthesia, and red cell lipids were extracted by the Bligh and Dyer procedure (17). The extracts were derivatised by treatment with boron trifluoride in methanol (14%) for 20 min at 70 C. The fatty acid methyl esters were extracted into hexane and analyzed on a Perkin Elmer F17 gas chromatograph with flame ionization detector and modified to take a support coated open tubular column (SP 1000). The injector and detector temperature was 300 C, and the fatty acid esters were analyzed at a temperature programmed at 0.5 C/min between 180 and 215 C. Samples and standards were quantitated by integration (Hewlett Packard 3380A).

RESULTS

Systolic blood pressures of two groups of rats over the period of maintenance on experimental diets are shown in Figure 1. After 2 weeks, rats receiving DHLA at 5.0% dietary energy established a significantly lower blood pressure than those fed the non-supplemented diet. The difference in blood pressure between these groups was maintained for a further 4 weeks at which time their diets were crossed over. Within 2 to 3 weeks of reversing the diets, the direction of blood pressure difference between these groups was reversed and levels were maintained to the end of study (Fig. 1). Mean blood pressure of rats receiving 5.0% energy as DHLA (143.5 ± 1.6 mm Hg) was consistent with normotension. Levels were similar to those measured under the same conditions in rats fed a stock pellet diet (142.8 ± 0.9 mm Hg) or a diet enriched in sunflower oil containing 23% energy as linoleic acid (142.0 ± 1.3 mm Hg).

One group of rats was fed the saturated fat-rich diet with 0.5% energy as DHLA throughout the 11-week study. Blood pressures of these rats were not significantly different from those fed the non-supplemented diet, either in the period before or after dietary crossover. At termination of the study the proportion of animals in each dietary group with blood pressures above 150 mm Hg was 75%, 67% and 0% for the non-supplemented diet and diets containing 0.5% or 5.0% energy as DHLA, respectively.

Food intake, measured weekly, was similar for rats on all three diets throughout the study. Mean consumption of calories per rat calculated from 24 measurements of food intake over the study was 68.5 ± 8.6, 68.9 ± 8.5 and 67.1 ± 6.4 kcal·24 h^{-1} for animals maintained on the non-supplemented diets and diets with 0.5% and 5.0% energy as DHLA respectively. These values were not significantly different (two sided Student t test).

Levels of PGE_1 in the urine of rats receiving 5.0% energy as DHLA at termination were significantly increased over levels in those receiving the other two diets ($p<0.002$). The measurements of PGE_1 ($\bar{x}$ ± s.e.m., pg.μmol creatinine^{-1} · kg body weight^{-1}) for the three groups of rats receiving diets containing 0%, 0.5% and 5.0% energy as DHLA at termination were respectively 92.4 ± 7.3; 83.5 ± 9.9, and 306.7 ± 54.2. The corresponding levels of PGE_2, 2250 ± 224; 1834 ± 218 and 2391 ± 390, were not significantly different for the three groups.

The composition of fatty acids in the red cell lipids of rats receiving the non-supplemented diet at termination was similar to that of rats receiving DHLA (5.0% energy) (Table 3). Both diets caused a marked depression in levels of 18:2ω6 in comparison with those of rats fed a stock pellet, reference diet. However, levels of the 20 and 22 carbon essential fatty acids (EFA) in the red cells of rats fed the experimental diets were not decreased with respect to levels produced by the pellet diet (Table 3). Feeding the experimental diets did not significantly affect the ratio 20:3ω9/20:4ω6 (Table 3).

TABLE 2

Cross Reactivity (%) of Antisera to PGE with Other Prostaglandins

PG	Antiserum PGE_1	Antiserum PGE_2
A_1	0.1	<0.1
A_2	<0.1	0.2
B_1	<0.1	<0.1
B_2	<0.1	<0.1
D_1	0.1	–
D_2	<0.1	–
E_1	100.0	3.2
E_2	15.0	100.0
$F_{1\alpha}$	0.2	<0.1
$F_{2\alpha}$	<0.1	0.1
dihydro-E_1	–	<0.1
dihydro-E_2	–	0.15
dihydro-keto-E_1	0.2	<0.1
dihydro-keto-E_2	<0.1	0.1

TABLE 3

Fatty Acid Composition (% by Weight) of Total Red Cell Lipids

	Diet at Termination		
	Non-supplemented	With DHLA (5.0% energy)	Reference[a] Diet
Fatty acid composition[b]			
16:0	25.48 ± 1.08	26.38 ± 0.56	22.38 ± 1.18
16:1	3.40 ± 0.48	2.87 ± 0.22	2.02 ± 0.16
18:0	9.30 ± 0.52	9.72 ± 0.30	9.88 ± 0.23
18:1	14.49 ± 1.06	11.45 ± 0.28	11.46 ± 0.99
18:2ω6	2.55 ± 0.14	2.19 ± 0.08	12.77 ± 0.89
20:3ω6	4.24 ± 0.36	6.56 ± 0.16	0.41 ± 0.07
20:4ω6	25.77 ± 1.69	26.56 ± 1.06	18.90 ± 0.57
22:4ω6	3.24 ± 0.24	3.21 ± 0.18	2.20 ± 0.21
22:5ω6	2.46 ± 0.24	1.86 ± 0.16	1.58 ± 0.38
20:3ω9	0.11 ± 0.05	0.24 ± 0.03	0.21 ± 0.06
20:3ω9/20:4ω6	0.006 ± 0.004	0.009 ± 0.002	0.011 ± 0.003

[a]Data are for animals of similar age and weight maintained on a certificated pelleted diet. This was a natural ingredient diet which contained 3.0% by weight of oils and provided the following fatty acids (wt % of diet): 16:0, 0.28%; 18:1, 0.85%; 18:2ω6, 0.50%, and 20:4ω6, 0.16%.

[b]Mean ± s.e.m., n=10.

DISCUSSION

The variations between the blood pressure of groups of rats fed the experimental diets are ascribed to differences in the composition of dietary fat, since similar total amounts of food were consumed in each case. The results are consistent with the evidence of others that diets enriched in saturated fat induce hypertension (1-3). They show for the first time that incorporating low levels of DHLA (5.0% energy) can counteract this effect.

It is unlikely that the absence of elevated blood pressures of rats fed the diet containing 5% energy as DHLA can be ascribed to the very small reduction in saturated fat in that diet. Nor can this be ascribed to the fact that the other diets were inducing a state of EFA deficiency for the following reasons: (1) the body weights of groups fed the DHLA-supplemented and non-supplemented diets at termination were not significantly different, and no other gross changes (e.g. skin lesions) characteristic of EFA deficiency were observed; (2) no increment in 20:3ω9/20:4ω6 or depression in total EFA was found in the red cell lipids of rats receiving the non-supplemented diet at termination; (3) renal excretion of PGE_2, which is reduced in EFA deficiency, was similar in rats fed diets with or without DHLA, and (4) the rapid response of blood pressure to changing diets is difficult to reconcile with the progressive development of the EFA-deficient state.

In the present work, addition of DHLA (5% energy) to the high fat diet resulted in an increase in renal excretion of PGE_1, but no change in excretion of PGE_2. In other studies (C.H. Hassall, S.J. Kirtland, unpublished work) we have found that a diet providing linoleic acid (6% energy) failed to counteract hypertension induced with saturated fat. We propose that DHLA exerts a direct antihypertensive action which is probably related to its conversion to prostaglandins.

Increased levels of PGE_1 in urine, as found in these experiments, have been reported previously for animals receiving DHLA or its ethyl ester (16, 18); this probably reflects increased production of PGE_1 by the kidney (14,19). It has been observed by others that impaired renal synthesis of prostaglandins is associated with a reduction in renin-secretion, vasodilation and natriuresis, effects which may be important contributing factors in hypertension (20). PGE_1 is potent as a stimulator of renin secretion and as a vasodilator and natriuretic agent (21-23); it has been shown to have hypotensive properties (22,24).

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REFERENCES

1. Iacono, J.M.; Judd, J.T.; Marshall, M.W.; Canary, J.J.; Dougherty, R.M.; Makin, J.F., and Weinland,

B.T. (1981) Prog. Lipid Res. 20, 349-364.
2. Smith-Barbaro, P.; Fisher, H.; Quinn, M.R., and Hegsted, D.M. (1980) Nutr. Rep. Int. 22, 759-770.
3. Macdonald, M.C.; Kline, R.L., and Mogenson, G.J. (1981) Can. J. Physiol. Pharmacol. 59, 872-875.
4. Rosenthal, J.; Simone, P.G.; and Silbergleit, A. (1974) Prostaglandins 5, 435-440.
5. Ten Hoor, F. and Van de Graff, H.M. (1978) Acta. Biol. Med. Ger. 37, 875-877.
6. Tobian, L.; Ganguli, M.; Johnson, M.A., and Iwai, J. (1982) Hypertension 4, Suppl. II, 149-153.
7. Hoffman, P.; Forster, W., and Markov, Ch. M. (1982) Prostaglandins Leucotrienes Med. 8, 151-156.
8. Alfin-Slater, R.B. and Aftergood, L. (1976) in Lipid Pharmacology (Paoletti, R. and Glucek, C.J., eds.) Vol. II, pp. 43-82, Academic Press, New York and London.
9. Brenner, R.R. (1981) Prog. Lipid Res. 20, 41-47.
10. Jeffcoat, R. (1979) in Essays in Biochemistry (Campbell, P.N. and Marshall, R.D., eds.) Vol. 15, pp. 1-36, Academic Press, London, New York.
11. Holman, R.T. and Johnson, S. (1981) Prog. Lipid Res. 20, 67-73.
12. Willis, A.L.; Hassam, A.G.; Crawford, M.A.; Stevens, P., and Denton, J.P. (1981) Prog. Lipid Res. 20, 161-167.
13. Stone, K.J.; Willis, A.L.; Hart, M.; Kirtland, S.J.; Kernoff, P.B.A., and McNicol, G.P. (1979) Lipids 14, 174-180.
14. Knapp, H.R.; Oelz, O.; Whorton, A.R., and Oates, J.A. (1978) Lipids 13, 804-808.
15. Gerold, M. and Tschirky, H. (1968) Arzneim. Forsch. 18, 1285-1287.
16. Kirtland, S.J. and Shawyer, C.R. (1981) 23rd International Conference on Biochemistry of Lipids, Odense, Denmark.
17. Bligh, E.G. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911-917.
18. Ellis, C.K.; Whorton, R.; Oelz, O.; Sweetman, B.J.; Wilkinson, G.R., and Oates, J.A. (1977) Fed. Proc. 36, 402.
19. Frohlich, J.C.; Wilson, T.W.; Sweetman, B.J.; Smigel, M.; Nies, A.S.; Carr, K.; Throck Watson, J., and Oates, J.A. (1975) J. Clin, Invest. 55, 763-770.
20. Weber, P.C.; Siess, W., and Scherer, B. (1980) Clin. Exp. Hypertension 2, 741-760.
21. Yun, J.C.H.; Kelley, G.D.; Bartter, F.C., and Smith, G.W. (1978) Life Sci. 23, 945-952.
22. Karim, S.M.M. (1972) in The Prostaglandins: Progress in Research pp. 166-223, Medical and Technical Publications Co., Ltd., Oxford and Lancaster.
23. Johnston, H.H.; Herzog, J.P., and Lauler, D.P. (1967) Amer. J. Physiol. 213, 939-946.
24. Hirose, M.; Nakamoto, T.; Nishida, Y.; Ogawa, K., and Yoshimura, M. (1978) Dokkyo J. Med. Sci. 5, 179-190.

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Brominated Fatty Acid Distribution in Tissues and Fluids of Rats Fed Brominated Vegetable Oils

JAMES F. LAWRENCE, RAJINDER K. CHADHA, FRANK IVERSON, PETER MCGUIRE and HENRY B.S. CONACHER,* *Food Research Division, Health Protection Branch, Ottawa, Ontario, Canada K1A OL2*

ABSTRACT

Rats dosed orally for four days with 0.24 g/kg body weight per day of brominated olive oil (BOO) or brominated sesame oil (BSO) were found to accumulate dibromostearic (DBS) acid (from BOO) and DBS and tetrabromostearic (TBS) acids (from BSO) in the liver, heart and adipose tissue. The metabolites, dibromopalmitic, and dibromomyristic acids (from BOO and BSO), as well as their tetrabromo-analogues (from BSO) were found as determined by gas chromatography with halogen specific detection and confirmed by gas chromatography-mass spectrometry. Blood contained no DBS, TBS or metabolites. However, inorganic bromide was observed in both blood and urine while none was observed in the feces. The latter contained significant quantities of both DBS and TBS but showed the absence of the four brominated metabolites.
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INTRODUCTION

Brominated vegetable oils (BVOs) are permitted in Canada and the USA as dispersing agents in citrus flavored soft drinks. Limited toxicology studies in the rat (1-3) with BVOs have shown an increased bromine content of the lipids. Tinsley and coworkers (4-6) have reported on bromine levels in heart and liver lipids of the rat following feeding of brominated corn oil or the monoglycerides of dibromo- or tetrabromo-stearate. Using a debromination reaction (7) followed by gas chromatography with flame ionization detection, they determined the brominated fatty acid composition of heart, liver and adipose tissue lipid. We report here the results of a similar study using brominated olive oil and brominated sesame oil as a source of brominated fatty acids. The methodology used was the transesterification-gas chromatography technique reported recently for the determination of BVOs in soft drinks (8). In addition to heart, adipose and liver lipids, urine, blood and feces were analyzed either for brominated fatty acids or inorganic bromide.

METHOD

Feeding Studies

Solutions of brominated olive oil (BOO) and brominated sesame oil (BSO) (Abbott Laboratories, Montreal, Canada) were prepared at a concentration of 30% (w/v) in corn oil (Mazola). Aliquots (0.2 ml) of these solutions were administered by oral intubation to male Wistar rats weighing approximately 250 g each. The dose represented about 0.24 g/kg body weight.

Groups of eight rats were dosed daily by gavage for four days with BOO (group 1), BSO (group 2) and corn oil (control group, given 0.2 ml per day). The animals were kept in metabolism cages and fed standard rat chow cubes ad libitum. Urine and feces were collected on a 24 hr basis. On day five all rats were sacrificed by ether anesthesia. Liver, heart and adipose tissue were removed for analysis. A portion of blood was heparinized and plasma obtained for analysis.

Tissue and Fluid Analysis

Lipids were extracted from the tissues and feces by the method of Bligh and Dyer (9). Suitable volumes of the extracts were transferred to transesterification flasks and subjected to acid methanolysis with 2% sulfuric acid in methanol/hexane (5:1, v/v) (8). Methyl pentadecanoate (C_{15}) was added as an internal standard for quantitation. After the reaction was complete the products were extracted into hexane exactly as described earlier (8). An aliquot of each of these solutions was analyzed by gas chromatography.

Gas Chromatography

A Varian 2100 gas chromatograph fitted with a 2m × 2mm id glass column packed with 3% OV-3 on Chromosorb W,HP, 80-100 mesh was used for the determinations. Detector and injector temperatures were 280 C. The column was temperature programmed from 150-280 C at 10°/min with a helium carrier gas flow rate

*To whom correspondence should be addressed.

of 30 ml/min. The effluent emerging from the column was split and fed to a flame ionization detector (FID) and a Coulson electrolytic conductivity detector in the halogen mode. The signals were recorded on the same chart to facilitate identification of unknown halogen compounds. Quantitation was performed by peak area using electronic integration.

The confirmation of di- and tetrabromomyristic and di- and tetrabromopalmitic acids was carried out by gas chromatography-mass spectrometry by high resolution repetitive scanning with a VG 12000 quadrupole mass spectrometer in the electron impact mode at a potential of 70 eV, interfaced to a Dani gas chromatograph. Since the molecular ions at 398, 426, 558 and 586 m/e were absent, the compounds were characterized by their fragments as shown in Table 1.

RESULTS AND DISCUSSION

Figure 1 shows gas chromatograms obtained with FID detection of liver lipid extracts from control, BOO and BSO treated rats. The presence of DBS and TBS in the treated groups can be seen. The detector was neither sensitive nor selective enough to detect the halogenated metabolites. Figure 2 compares chromatograms

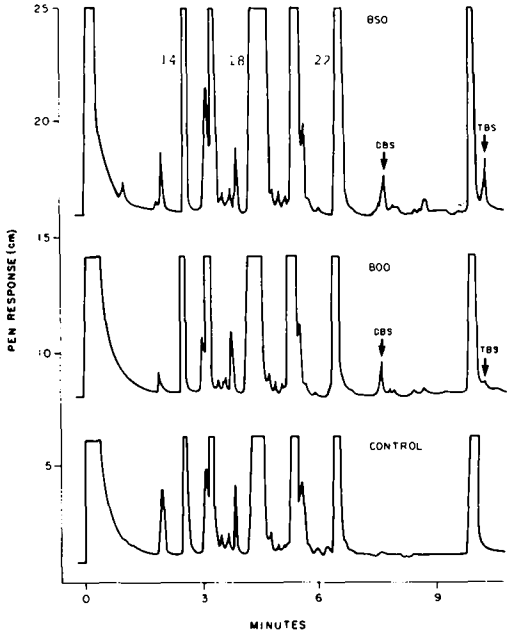

FIG. 1. A comparison of three typical gas chromatograms (FID detection) of liver lipid extracts from BSO, BOO and control rats. Conditions as described in the text. Peak numbers in the chromatogram refer to carbon chain lengths of non-halogenated fatty acids present in the samples.

TABLE 1

Mass Spectrometric Characterization of Brominated Fatty Acid Methyl Esters

Fragment	DBM-Me ($C_{15}H_{28}O_2Br_2$)	DBP-Me ($C_{17}H_{32}O_2Br_2$)	DBS-Me ($C_{19}H_{36}O_2Br_2$)	TBM-Me ($C_{15}H_{26}O_2Br_4$)	TBP-Me ($C_{17}H_{30}O_2Br_4$)	TBS-Me ($C_{19}H_{34}O_2Br_4$)
Molecular weight	398	426	454	554	582	610
$-Br$	319,321	347,349	375,377	475,477	503,505	531,533
$-HBr, -OCH_3$	287,289	315,317	343,345	443,445	471,473	499,501
$-HBr, -Br$	239	267	295	395	423	451
$-2HBr, -OCH_3$	207	235	263	363	391	399
-209	189	217	245	–	–	–
$CH_2{=}C(OH)OCH_3$	74	74	74	–	–	–

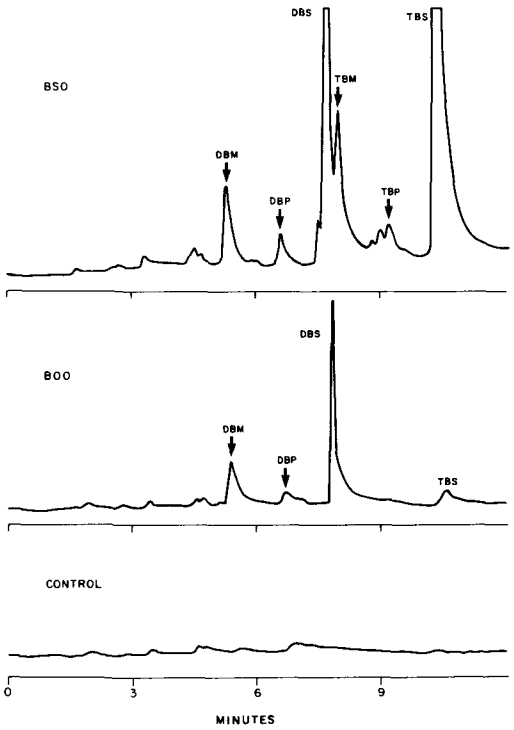

FIG. 2. A comparison of three typical gas chromatograms (electrolytic conductivity detection) of the same liver lipid extracts as shown in Figure 1. Conditions as described in the text.

obtained with conductivity detection of liver lipid extracts from the three groups. It readily can be seen that this detector is much more suited to brominated fatty acid analysis than is the FID. No halogenated fatty acid peaks are seen in the control liver. The DBS peak from the BOO and the DBS and TBS peaks from the BSO treated animals are readily apparent. In addition, peaks corresponding to shorter chain-length halogenated compounds also are present. Four peaks, indicated in the chromatograms, were confirmed by gas chromatography-mass spectrometry to be dibromomyristic acid (DBM), dibromopalmitic acid (DBP), tetrabromomyristic acid (TBM) and tetrabromopalmitic acid (TBP), suggesting metabolism by β-oxidation resulting in 2-carbon fragments being cleaved down to a C_{14} chainlength as earlier was reported by Conacher (10). No C_{12} brominated acids were observed. Jones et al. (5) reported similar findings. However, they were unsure about the presence of C_{12} acids due to the possible loss of these through the analytical technique they used. Taken together, all the available data support metabolism to C_{16} and C_{14} but not to C_{12} acids. Table 2 compares the dibromo-metabolite concentrations in the liver, heart and adipose tissue. In the liver and adipose tissue, DBM was the predominant metabolite while in the heart DBP was the major metabolite. This was observed for both BOO and BSO groups. These results differ from those reported by Jones et al. (5), who fed brominated corn oil or a mixture of monoglycerides of DBS or TBS. They observed that DBP predominated in the heart and adipose tissue but not in the liver. In general the two metabolites in the present work represented 10-20% of the dibrominated fatty acid content in the three tissues analyzed. Total dibromo-fatty acids were greatest in the heart, an observation also made by Jones et al. (5). The relative concentration of dibromo-fatty acids in the heart and liver tissues was significantly higher for BSO compared to BOO when the results were corrected for differences in DBS content of the original oils (BOO contained 74.6% DBS, while BSO contained 42.7% DBS). No difference was observed for adipose tissue.

TBS was found only in the liver tissues of the BSO group (Table 2). None was detected in

TABLE 2

Brominated Fatty Acids in Rat Tissues[a]

	Liver			Heart			Adipose		
Substance	Control	BOO	BSO	Control	BOO	BSO	Control	BOO	BSO
% Lipid	2.64	2.64	3.23	1.74	1.73	1.91	4.8	4.7	3.5
% DBFA[b]	–	0.88	0.69	–	1.45	1.12	–	0.91	0.51
DBS[c]	–	81.8	85.7	–	81.0	92.2	–	85.4	89.8
DBP	–	5.1	3.9	–	12.7	6.9	–	2.2	0.3
DBM	–	13.0	10.3	–	6.3	0.9	–	12.3	9.9
% TBS	–	–	0.93	–	–	–	–	–	–

[a]Averages of 8 animals.

[b]Per cent dibromo-fatty acids in the extracted lipid.

[c]Relative per cent of DBS, DBP and DBM in total DBFA.

the heart or adipose tissue. This observation is similar to that made by Jones et al. (5) in their study where TBS was shown to accumulate in the liver to a far greater extent than either heart or adipose. In this study the major tetrabromo metabolite observed in the liver was TBM (at about 10-20% of the TBS level). TBP was found to be present but at a level about 1% of that of TBS.

BOO had no effect on the tissue lipids while BSO caused an increase in per cent lipid content of the liver and heart. These effects are different from those of Jones et al. (5); they reported a substantial increase in lipid content of heart and liver with brominated corn oil and the monoglycerides of DBS and TBS.

Chromatographic analysis of blood samples showed the absence of brominated fatty acids. However, the average inorganic bromide content was determined to be 0.15 mg/ml plasma for the control group, 0.63 mg/ml and 0.69 mg/ml for the BOO and BSO groups, respectively, resulting in net increases of 0.48 mg/ml for the BOO group and 0.54 mg/ml for BSO. When corrected for different bromine content of the original oils (BOO contains 83% of the bromide level of BSO) the difference in the two treated groups becomes insignificant.

Table 3 shows the quantity of bromide excreted in the urine over the duration of the study. When plotted graphically it appears that a steady-state excretion of bromide has not been quite reached after four days for either treated group. When corrected for differences in bromide content of the original oils, the excretion rates and levels are essentially the same for both treated groups. The urine was not analyzed for brominated fatty acids.

Only the unchanged DBS and TBS were found in the feces. No fatty acid metabolites were observed nor was any ionic bromide detected. There also was no apparent trend in the levels of DBS or TBS observed with time. However, as can be seen in Table 4, the ratio of total DBS:TBS found in the feces of the BOO group is higher by a factor of about 2.4 compared to their ratio in the original oil. An increase of 1.6 fold was found for the BSO group. This indicates that TBS appears to be absorbed to a greater extent than DBS for the two oils studied, or it is degraded to unknown products preferentially in the small intestine.

TABLE 3

Bromide in Rat Urine[a]

Day	BOO	BSO
1	1.89	4.24
2	4.86	6.26
3	7.25	7.51
4	7.48	9.05
TOTAL:	21.48	27.06

[a]mg total per day, corrected for control values (average 1.11 mg per day).

TABLE 4

Brominated Fatty Acids in Feces[a]

Group/Day	DBS (mg)	TBS (mg)	Feces ratio	Oil ratio	Difference[b] by factor of:
BOO/1	6.6	0.5	13.2	6.3	2.1
2	7.9	0.5	15.8	6.3	2.5
3	2.2	0.1	22.0	6.3	3.5
4	7.7	0.5	15.4	6.3	2.4
TOTAL	24.4	1.6	15.3	6.3	2.4
BSO/1	1.4	1.2	1.2	0.9	1.3
2	2.3	1.5	1.5	0.9	1.7
3	1.0	0.7	1.4	0.9	1.6
4	2.0	1.5	1.3	0.9	1.4
TOTAL	6.7	4.9	1.4	0.9	1.6

[a]Averages of four rats; mg total per day.

[b]Factor = feces ratio/oil ratio.

REFERENCES

1. Gaunt, I.F.; Grosov, P., and Gangolli, S.D. (1971) Fd. Cosmet. Toxicol. 9, 1-11.
2. Gaunt, I.F.; Gangolli, S.D., and Crampton, R.F. (1971) Fd. Cosmet. Toxicol. 9, 13-19.
3. Munro, I.C.; Hand, B.; Middleton, E.J.; Heggtveit, H.A., and Grice, H.D. (1972) Toxicol. Appl. Pharmacol. 22, 432-439.
4. Jones, B.A.; Tinsley, I.J., and Lowry, R.R. (1983) Lipids 18, 319-326.
5. Jones, B.A.; Tinsley, I.J., and Lowry, R.R. (1983) Lipids 18, 327-334.
6. Wilson, G.R.; Tinsley, I.J., and Lowry, R.R. (1983) Lipids 18, 661-663.
7. Frankel, J.S., and Brown, J.B. (1943) J. Am. Chem. Soc. 65, 415-418.
8. Lawrence, J.F.; Chadha, R.K., and Conacher, H.B.S. (1983) J. Assoc. Offic. Anal. Chem. 66, 1385-1389.
9. Bligh, E.G. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 747-56.
10. Conacher, H.B.S. Presented at the 70th Annual Meeting of the American Oil Chemists' Society, San Francisco, April 29, 1979.

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The Effect of 3-Methylindole on the Uptake and Incorporation of ^{14}C-Choline into Phospholipids in Lung Tissue Slices

JAMES B. KIRKLAND and **TAMMY M. BRAY***, *Department of Nutrition, University of Guelph, Guelph, Ontario, Canada N1G 2W1*

ABSTRACT

3-Methylindole (3MI) is the causative agent in the development of acute bovine pulmonary edema. Microscopic studies revealed a structural disruption in the lamellar bodies of type II cells, indicating an abnormal metabolism of phospholipid in the lung of 3MI treated animals. In the present study, lung slices from 4 goats were used to investigate the changes in phosphatidylcholine metabolism induced by 3MI. Eighteen slices were cut from each healthy lung and divided into control and 3MI groups. After a 4-hr pretreatment with 3MI (.19 or .57 mM) or carrier, the level of incorporation of ^{14}C-choline into phosphatidylcholine, sphingomyelin and their water soluble intermediates was studied. The uptake of ^{14}C-choline and its incorporation into phosphatidylcholine and sphingomyelin was depressed by 3MI treatment. In the water soluble fractions, the radioactivity increased in free choline and CDP-choline, while it decreased in P-choline. This suggests that choline kinase and the P-choline transferases have become relatively more rate limiting and may play a role in the depressed de novo synthesis of phosphatidylcholine induced by 3MI.

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INTRODUCTION

Acute pulmonary edema is a naturally occurring, chemically induced lung disease, which is known to affect cattle, sheep and goats. The causative agent is 3MI, a ruminal fermentation product of tryptophan (1). The production of 3MI in the rumen is induced when the animal is faced with a sudden increase in dietary protein. Humans are exposed to 3MI through cigarette smoke (2) and the fermentation of tryptophan in the lower gut (3), although the potential risk from this exposure has not been investigated.

3MI is lipophilic and has been shown to interact with membrane lipids and alter membrane function in vitro (4,5). The development of the disease is not due to these direct effects, but is dependent on the metabolism of 3MI by the mixed function oxidases (MFO) located in the smooth endoplasmic reticulum (SER). The severity of the lung damage is lessened when the MFO system is inhibited (6). Reactive metabolites formed by the MFO metabolism of 3MI have been shown to bind covalently to microsomal proteins (7).

Microscopic studies of the early stages of 3MI induced lung injury in goats demonstrated that the toxic effects are rapid and cellular selective. The major effects are on Clara cells and alveolar Type I cells. Interstitial and alveolar edema also are present within a few hours after 3MI administration (8). After the initial damage, alveolar type II cells proliferate, but electron micrographs reveal some striking abnormalities in these cells. At 72 hr after 3 MI infusion, the lamellar bodies are devoid of the usual structure of transverse ribs. These organelles are the storage and secretory granules of surfactant in type II cells (9). With 3MI treatment the surfactant phospholipid (PL) in lamellar bodies are replaced by neutral lipids (NL) (10).

These observations indicated that 3MI metabolism may cause an inhibition of PL synthesis. Phosphatidylcholine (PC) is the major component of surfactant and an important structural component of lung membranes. The major synthetic route for PC in the lung is reported to be the CDP-choline pathway (11). The de novo synthesis of PC is dependent on the uptake of choline from the blood and its metabolism through the intermediate compounds phosphorylcholine (p-choline) and cytidine diphosphate choline (CDP-choline). 3MI may inhibit the de novo synthesis of PC by inhibiting the uptake of choline from the medium, or by inhibiting one or more of the required enzymes. In the present study we have investigated the effect of 3MI on the uptake of ^{14}C-choline by goat lung slices and its incorporation into PC, sphingomyelin (SM) and their water soluble intermediates.

MATERIALS AND METHODS

Four healthy crossbred male goats (Farr's

*To whom correspondence should be addressed.

Farm Service, Guelph, Ontario) from 6-12 months of age were used in this study. They were maintained ad libitum on alfalfa hay with a concentrate supplement (22% protein) and tap water. The animals were killed with a captive bolt gun, and the lungs were removed and placed on ice.

[Methyl-^{14}C]-choline (50.5 mCi/mmol) was obtained from New England Nuclear, Boston, Massachusetts; 3MI from Sigma Chemical, St. Louis, Missouri; silica gel H from Analabs, North Haven, Colorado, and fluorescein from Eastman Organic Chemicals, Rochester, New York. Standards for PC and SM were obtained from Sigma Chemical, and ^{14}C-labelled standards for P-choline and CDP-choline were obtained from New England Nuclear. Other reagents were obtained from Fisher Scientific Ltd., Toronto, Ontario.

Eighteen slices (1 mm thickness) were cut from each lung using 2 fixed microtome blades. The disposable microtome blades were held a distance of 1 mm apart by the placement of a small brass strip at each end, held together by small bolts. When this was used to cut through a section of lung, it produced clean slices of consistent thickness. New blades were used for each lung. The lung was divided into right and left halves. The lower third portion of each half was removed, and the upper portion was bisected vertically. Three slices were cut from each of the 6 sites. The slices from each site were divided into control and 2 treatment groups (3MI at .19 and .57 mM), reducing the variability associated with different areas of the lung. The slices were weighed immediately following cutting, and one slice of 100-200 mg wet weight was placed in each flask. The 25 ml flasks contained 4 ml of Krebs Ringer bicarbonate buffer (pH 7.4), with additional glucose (10 mM), pyruvate (1 mM) and a mixture of 21 amino acids (12). The concentrations of amino acids were modified from those used by Eagle (13). The flasks were placed in a shaking water bath at 37 C and gassed continuously with 95% O_2 and 5% CO_2. After a 4-hr incubation with carrier (15 μl ethanol), .57 mM 3MI or .19 mM 3MI, 1 μCi of ^{14}C-choline was added to each flask. Incubation was continued for 2 hr. The slices were removed, washed and homogenized in 5 ml of chloroform:methanol (2:1, v/v), using an Ultra Turrax homogenizer (8 mm diameter). The incorporation of ^{14}C-choline was tested from 4-6 hr after slicing and found to be linear. The stability of the lung slices was tested by comparing the incorporation from 0-2 hr after slicing to that from 4-6 hr. These levels were found to be identical. One ml of water was added to the tissue homogenate to create a 2-phase system. The chloroform phase was removed and evaporated to dryness under a stream of N_2. The lipids were redissolved in 100 μl of chloroform:methanol (2:1, v/v), and spotted on plates coated with silica gel H (.50 mm). The plates were developed in a solvent system consisting of chloroform: methanol:water (50:40:5, v/v/v) and sprayed with a saturated solution of fluorescein in methanol:water (1:1, v/v). This is a modification of the method of Skipski et al. (14). The plates were dried, and the spots visualized under UV light. The bands containing the various lipid fractions were scraped into vials and resuspended in 1.5 ml of water. Scintiverse counting fluid (Fisher Scientific Ltd.) was added, and the radioactivity was measured in a Searle liquid scintillation counter.

The water soluble intermediates in the water: methanol phase of the tissue homogenate were separated on Watman #1 paper. The papers were developed in a descending system using n-butanol:acetic acid:water (5:1.6:4, v/v/v). The samples were separated over a distance of 18 cm. The papers were cut into strips with a width of 1 cm, and placed in vials with a toluene based counting fluid containing 5g/l POP and 50 mg/l POPOP. The radioactivity was determined and totalled within each peak. The following Rf values were obtained for the intermediates; CDP-choline .25, P-choline .43, and choline .75. An aliquot of the incubation medium was counted to calculate the uptake of ^{14}C-choline by the slices.

The level of incorporation of radioactivity into each fraction was averaged across the 6 slices in each group. The effect of treatments was expressed as a percentage change from the control values, and this difference was assessed in a group of 4 goats using a paired Student's t-test (15). Some data also was evaluated using the sign test (15).

RESULTS

The uptake of choline into the lung slices was calculated by measuring the disappearance of radioactivity from the medium (Table 1). This figure includes the choline present in all the intermediates and end products of PC and SM biosynthesis, as well as the choline which is used in other metabolic pathways or oxidized to CO_2. 3MI, at .57 mM, caused a 38% depression in the uptake of choline from the medium. The lower level of 3MI (.19 mM) also caused a depression, but this effect was not statistically significant.

Table 2 summarized the effect of 3MI on the incorporation of ^{14}C-choline into the

TABLE 1

The Effect of 3-Methylindole (3MI) on the Uptake of ^{14}C-Choline by Goat Lung Slices

	Uptake of ^{14}C-choline*	
	(dpm/100 mg tissue)	(% depression ± SD)
Control	434,900	0
3MI (.19 mM)	338,490	-22.0 ± 11.0
3MI (.57 mM0	270,080	-37.7[a]± 7.1

*Values given are the means from 4 goat lungs, 6 slices per lung.
[a]The value is significantly different ($p<0.05$) when the treatment group is compared with its paired control group by the Student's paired t-test.

TABLE 2

The Effect of 3MI on the Incorporation of ^{14}C-Choline into the Choline Containing Lipids

	Sphingomyelin*		Phosphatidylcholine*	
	dpm/100 mg tissue	% depression (± SD)	dpm/100 mg tissue	% depression (± SD)
Control	2,200	0	179,130	0
3MI (.19 mM)	1,130	-45.6[a] (±15.9)	112,780	-36.8[a] (±16.0)
3MI(.57 mM)	485	-75.7[a] (±10.0)	75,850	-57.0[a] (±11.6)

*Values given are the means from 4 goat lungs, 6 slices per lung.
[a]The value is significantly different ($p<0.05$) when the treatment group is compared with its paired control group by the Student's paired t-test.

choline-containing lipids. 3MI, at both .57 and .19 mM concentrations, caused significant depressions in the incorporation of ^{14}C-choline into PC and SM. The higher level of 3MI caused a 76% depression in the incorporation of ^{14}C-label into SM and a 57% depression in the incorporation into PC. Although the effect of 3MI was greater in the SM fraction, the incorporation of ^{14}C-label into the PC fraction was greater by 2 orders of magnitude. The lung slices have a much greater capacity for the incorporation of choline into PC than they have for SM.

Table 3 summarized the effect of 3MI on the accumulation of ^{14}C-radioactivity in the water soluble intermediates of PC and SM synthesis. In the control slices, the greatest accumulation of ^{14}C-label occurred in P-choline, with slightly less appearing in free choline and only trace amounts in the CDP-choline fraction. Pretreatment with .57 mM 3MI caused a 21% increase in free ^{14}C-choline within the slice, while the incorporation of label into the P-choline fraction was depressed by 48%. Although the level of radioactivity in CDP-choline more than doubled with 3MI treatment, the response was variable and not statistically significant using the Student's t-test. As 3MI increased the radioactivity in this fraction in all of the lungs used, the sign test indicates that this is a significant increase, although it does not provide information on the magnitude. 3MI, at the lower concentration of .19 mM, caused a similar trend, but the differences were not statistically significant by the paired Student's t-test.

DISCUSSION

3MI (.57 mM) caused large depressions in the incorporation of ^{14}C-choline into the lipids SM (-76%) and PC (-57%). In our previous work using ^{14}C-acetate as a label, similar results were obtained, even though these precursors are incorporated via distinctly separate pathways. 3MI (.57 mM) depressed ^{14}C-acetate incorporation into SM by 72% and into PC by 46%, but did not affect its incorporation into neutral lipid (12).

The synthesis of PC and SM is dependent on the uptake of choline from the medium, and its metabolism through the intermediates P-choline and CDP-choline. 3MI may inhibit the incorporation of labelled choline into PC and

TABLE 3

The Effect of 3MI on the Incorporation of ^{14}C-Choline into Water Soluble Intermediates

	Choline*	P-Choline*	CDP-Choline*
Control			
dpm/100 mg tissue	68,050	72,270	3,540
3MI (.19 mM)			
dpm/100 mg tissue	71,720	47,100	7,440
% change	+ 5.4	-35.2	+138.7
(± SD)	(±12.7)	(±20.1)	(±92.6)
3MI (.57 mM)			
dpm/100 mg tissue	81,550	37,810	8,000
% change	+20.7[a]	-47.9[a]	+129.0[b]
(±SD)	(± 8.1)	(± 8.2)	(±74.4)

*Values given are the means from 4 goats, 6 slices per lung.

[a]The value is significantly different ($p<0.05$) when the treatment group is compared with its paired control group by the Student's paired t-test.

[b]The value is a significant increase ($p<0.05$) when the treatment group is compared with its paired control group using the sign test.

SM by inhibiting the uptake of choline by the lung slice, or by inhibiting one or more of the required enzymes. These are, in sequence, choline kinase, P-choline cytidyl transferase, and the P-choline transferases. P-choline cytidyl transferase is generally thought to be rate limiting (16), and this is supported by the accumulation of ^{14}C-label in the P-choline and free choline fractions of the control slices.

The decreased incorporation of ^{14}C-choline into PL may also be due to differences in pool sizes of intermediates, with no change in the synthesis of PC or SM. This is unlikely for several reasons. Special care was taken to ensure that the 3 slices in each group were from the same location in the lung. Although differences in pool sizes may develop during the pretreatment period, the 2-hr exposure to ^{14}C-choline should largely equilibrate the specific activities in these pools. Also, if this effect was an artifact of different pool sizes of choline intermediates, the results from the ^{14}C-acetate tracer study would not have indicated the same depressions in lipid synthesis (12). Lastly, if the decreased incorporation of label into the lipids was due to the dilution of ^{14}C-choline, one would expect a consistent effect throughout the pathway. This is not the case, as 3MI increases the radioactivity in free choline and CDP-choline while decreasing that in P-choline and the final products.

At .57 mM, 3MI did cause a large depression in the uptake of ^{14}C-choline by the lung slices (Table 1). These results implied that 3MI on the plasma membrane may inhibit the uptake of choline and limit the synthesis of PC and SM. The level of free ^{14}C-choline within the slice, however, was increased 21% by treatment with .57 mM 3MI (Table 3). The depressed PC and SM synthesis induced by 3MI is not, therefore, caused by a limited concentration of the substrate, choline, within the tissue slice. The depression in choline uptake by the slice does not appear to be caused exclusively by a direct effect on the plasma membrane or transport mechanisms. The accumulation of choline within the slice may have a feedback inhibitory effect on the uptake of choline from the medium.

The accumulation of ^{14}C-label in the water soluble intermediates was measured to investigate the mechanism by which 3MI depresses PC and SM synthesis. The accumulation of free ^{14}C-choline, and the 48% depression in ^{14}C-P-choline (Table 3), indicates that the enzyme required for this conversion, choline kinase, is inhibited by 3MI treatment. In spite of the 48% depression in ^{14}C-P-choline, the level of radioactivity in CDP-choline was increased with 3MI treatment. This suggests that 3MI is not limiting the activity of the second enzyme, P-choline cytidyl transferase, which is generally thought to be rate limiting. Since the incorporation of label into the final products, PC and SM, is decreased, the increase in ^{14}C-CDP-choline probably is due to an inhibition of the terminal enzymes, the P-choline transferases.

In conclusion, the present studies have shown that 3MI treatment causes a decrease in the uptake of choline from the medium and its incorporation into the lipids PC and SM. The effect of 3MI on the biosynthesis of PC is depicted in Figure 1. With 3MI treatment, the radioactivity in free choline and CDP-choline is increased, while it is decreased in P-choline. This suggests that choline kinase and the P-choline transferases have become relatively more rate limiting and may play a role in the

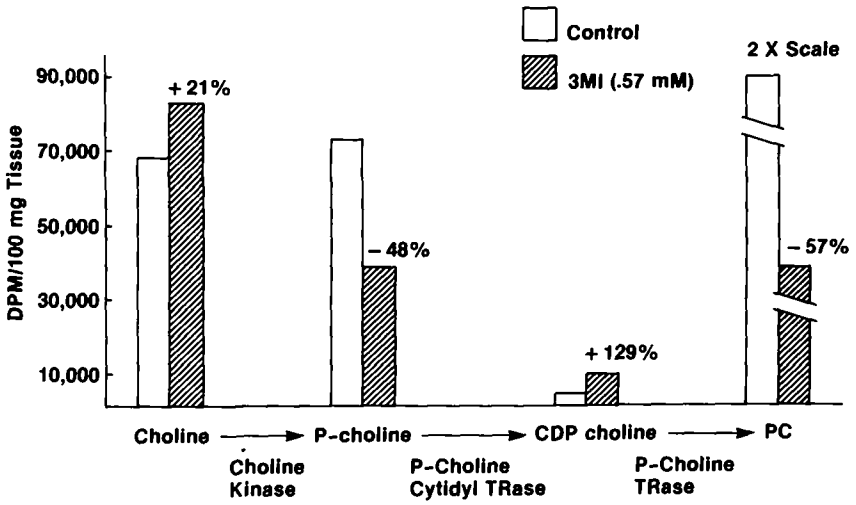

FIG. 1. The effect of 3MI on the incorporation of ^{14}C-radioactivity in the intermediates and end products of PC synthesis in goat lung tissue slices after incubation with ^{14}C-choline. The enzymes involved in the synthesis of PC are listed below the intermediates.

decreased de novo synthesis of PC. The accumulation of radioactivity in intermediate compounds does not give quantitative information on the changes in enzyme activity, but does suggest that there is inhibition at one or more points in this pathway. More detailed work, characterizing the changes in enzyme activity, is required in this area.

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REFERENCES

1. Carlson, J.R.; Yokoyama, M.T., and Dickinson, E.O. (1972) Science 175, 298-299.
2. Hoffman, D., and Rathcamp, G. (1970) Anal. Chem. 42, 366-370.
3. Yokoyama, M.T., and Carlson, J.R. (1979) Am. J. Clin. Nutr. 32, 173-178.
4. Rogers, K.S. (1969) Proc. Soc. Exp. Biol. Med. 130, 1140-1142.
5. Bray, T.M., and Carlson, J.R. (1975) Proc. Soc. Exp. Biol. Med. 148, 875-879.
6. Bray, T.M., and Carlson, J.R. (1979) Am. J. Vet. Res. 40, 1268-1272.
7. Bray, T.M., and Carlson, J.R. (1979) Fed. Proc. 38, 1329.
8. Bradley, B.J., and Carlson, J.R. (1980) Am. J. Pathol. 99, 551-559.
9. Gil, J., and Reiss, O.K. (1973) J. Cell. Biol. 68, 152-171.
10. Atwal, O.S., and Bray, T.M. (1981) Am. J. Pathol. 105, 255-263.
11. Van Golde, L.M.G. (1976) Am. Rev. Res. Dis. 114, 977-1000.
12. Kirkland, J.B., and Bray, T.M. (1983) Proc. Soc. Exp. Biol. Med. 175, 30-34.
13. Eagle, H. (1955) Proc. Soc. Exp. Biol. Med. 89, 362-364.
14. Skipski, V.P.; Peterson, R.F., and Barkley, M. (1964) Biochem. J. 90, 374-378.
15. Steel, R.D.G., and Torrie, J.H. (1960) Principles and Procedures of Statistics, p. 78 and p. 401, McGraw Hill, London.
16. Infante, J.P. (1977) Biochem. J. 167, 847-849.

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Characterization of a Cytosolic Protein Inhibiting Lysosomal Acid Cholesteryl Ester Hydrolase

MITSUO TANAKA*, RYOOJI YONEKURA, TOSHIHIRO IIO and TOSHIKAZU TABATA,
Showa College of Pharmaceutical Sciences, 1-8, Tsurumaki-5-chome, Setagaya-ku, Tokyo, Japan

ABSTRACT

An inhibitor of lysosomal acid cholesteryl ester hydrolase (Acid CEH), (EC 3.1.1.13) was found in the cytosolic fraction of rat liver and various other tissues. The extent of the inhibitory effect was dependent on the concentration of the cytosolic protein. The Acid CEH inhibitor was heat-labile, non-dialyzable, and its inhibitory activity significantly decreased by trypsin or chymotrypsin digestion, but not by lipase digestion. The inhibitor had no effect on the activity of cathepsin D, β-glucuronidase and acid phosphatase, which are other enzymes found in lysosomes. The present findings suggest that the inhibitor may be involved in the regulation of the hydrolysis of cholesteryl esters in lipoproteins that have been transferred into the liver.
Lipids 19:714-720, 1984.

INTRODUCTION

A lysosomal Acid CEH from rat liver was first reported by Stoffel and Greten (1), and was characterized and partially purified by Brown et al. (2). It exhibited a molecular weight of about 60,000 and was shown to be a glycoprotein with sulfhydryl groups essential for enzyme activity (3,4). It is well known that Acid CEH is required for hydrolysis of endogenous and exogenous cholesteryl esters in several tissues, including liver, spleen and aorta.

There is a marked accumulation of cholesterol and cholesteryl esters in the arterial wall during the pathogenesis at atherosclerosis (5), and it has been suggested that the accumulation of cholesteryl esters may be responsible for the change in lysosomal membranes which accompanies the decrease in Acid CEH activity (6).

We found recently that lysosomal Acid CEH activity in hypercholesterolemic rat liver decreased markedly in comparison with that of normal rat (Tanaka, M., unpublished data). However, the relationship between lysosomal Acid CEH activity and any physiological role in the regulation of cellular cholesteryl esters metabolism has not yet been established.

In the course of an investigation of the characteristics of lysosomal Acid CEH, we found a cytosolic protein in rat liver that has an inhibitory effect on this enzyme. In this paper, we present a partial characterization of the cytosolic inhibitor of Acid CEH.

MATERIALS AND METHODS

Chemicals and Radiochemicals

Cholesteryl [1-^{14}C] oleate (specific activity 58.6 mCi/m mole) was purchased from New England Nuclear Corp. Trypsin, chymotrypsin, trypsin inhibitor, phenolphthalein glucuronide, phenylphosphate, hemoglobin, chymotrypsinogen and α-N-benzoyl-DL-arginine-2-naphthylamide hydrochloride (BANA) were purchased from Sigma Chemicals Co. Ltd.

Preparation of Lysosomal and Cytosolic Fraction

Young male Sprague-Dawley rats weighing 150-200 g were used for all studies. For the preparation of lysosomal and cytosolic fractions from various tissues, the method of Brecher et al. (7) was followed. Rats were killed by guillotine and livers were perfused with ice-cold 1.15% KCl solution at 4 C. The tissues were homogenized in 8 volumes of ice-cold 0.25 M sucrose/1 mM EDTA/0.01 M Tris-HCl buffer (pH 7.5). The homogenate was centrifuged at 1,000 xg for 10 min and the resulting supernatant was centrifuged at 3,300 xg for 20 min. The 3,300 xg supernatant solution was centrifuged at 12,000 xg for 35 min. The pellet was rehomogenized in 0.25 M sucrose solution and recentrifuged at 12,000 xg for 20 min. The resulting pellet was resuspended in 0.25 M sucrose/0.01 M Tris-HCl buffer (pH 7.4) and used for enzymatic study. The original 12,000 xg supernatant solution was recentrifuged at 105,000 xg for 60 min to obtain the cytosolic fraction.

Acid Cholesteryl Ester Hydrolase Assay

The activity of Acid CEH was measured by the method of Brecher et al. (7). Benzene solutions of cholesteryl oleate and cholesteryl [1-^{14}C] oleate were mixed, and the benzene was evaporated under nitrogen. Saline solution with 0.5% albumin was added, and the mixture was sonicated 3 times for 10 sec. The standard incubation mixture usually

*To whom correspondence should be addressed.

contained 0.69 nmol of cholesteryl [1-^{14}C] oleate (0.04 μCi) in 0.15 M acetate buffer (pH 4.5) and 50 μg of lysosomal protein in a final volume 0.3 ml. The tubes were incubated at 37 C for 30 min. The reaction was terminated by addition of 3.0 ml of benzene/chloroform/methanol mixture (1:0.5:1.2, v/v/v) containing unlabeled oleic acid (0.1 mM) as carrier. NaOH (0.6 ml of 0.3 M) was then added. The solution was mixed for 25 sec on a vortex mixer and centrifuged for 10 min at 3,000 rpm. The amount of liberated [1-^{14}C] oleate in the upper aqueous phase was determined by adding 0.5 ml aliquot to 10 ml of Aquasol 2 liquid scintillation mixture and counting the samples in an Aroka LSC 900 liquid scintillation counter.

Gel Filtration Chromatography

The cytosolic fraction was further treated by reducing the pH from 7.4 to 5.1 with acetic acid. The mixture was left at 4 C for 2 hr and then centrifuged at 15,000 xg for 30 min. The supernatant was dialyzed against 50 volumes of 0.01 M phosphate buffer (pH 7.5). The dialyzed supernatant was applied to a column (3 x 60 cm) of Sephacryl S-200 (Pharmacia Fine Chemicals). The column was equilibrated with 0.01 M phosphate buffer (pH 7.5) containing 0.1 M NaCl. Fractions of 5 ml were collected and 50 μl of each fraction was assayed for activity with respect to inhibition of Acid CEH and thiol proteinase (cathepsin B).

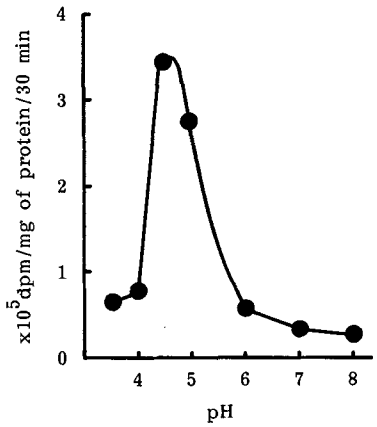

FIG. 1. Effect of pH on hydrolysis of cholesteryl esters by liver lysosomal fraction. Incubation in the pH range of 3-5 was carried out in 0.15 M sodium acetate buffer. For pH 6 and the pH range 7-8, incubations were done in 0.15 M phosphate buffer and 0.15 M Tris-HCl buffer, respectively. Standard assay conditions were used as described in Materials and Methods.

Assay of Lysosomal Marker Enzymes

Cathepsin D was determined by a modification of the procedure of Hirado et al. (8). The reaction mixture (1.0 ml), containing 20 mg hemoglobin, 1.0 M formate buffer (pH 3.0) and 0.5 ml enzyme solution, was incubated at 45 C for 30 min. The reaction was stopped by adding 3% TCA and the solution mixed for 30 sec and centrifuged for 10 min at 3,000 rpm. The amount of reaction products in the supernatant was assayed by the method of Lowry et al. (9). Cathepsin B was assayed by the method of Lenney et al. (10), using BANA as substrate. β-Glucuronidase was assayed with phenolphthalein glucuronide as substrate. Phenolphthalein liberated from the substrate was measured by the method of Gianetto et al. (11). Acid phosphatase was measured using phenylphosphate as a substrate essentially as described previously (12). Protein concentration was assayed by the method of Lowry et al. (9).

RESULTS

Optimal Assay Conditions for Lysosomal Acid CEH Activity

The effect of pH on lysosomal Acid CEH activity was examined in the range from 3 to 8, using acetate, phosphate and Tris-HCl buffers. Under our assay conditions, the highest rate of Acid CEH activity was observed at pH 4.5 in sodium acetate buffer (Fig. 1), and its activity was negligible below pH 4.0 or above pH 6.0. The rate of hydrolysis was linear for 60 min, and good proportionality with enzyme concentration was observed up to about 50 μg of the lysosomal protein.

Effect of Various Metals on Acid CEH Activity

Recent studies demonstrated that lysosomal Acid CEH was affected by several cations (13). We found (Fig. 2a, 2b) the enzyme was significantly inhibited at 1.0 mM concentrations of $FeCl_2$, $FeCl_3$ and $ZnCl_2$. In addition, $MnCl_2$ at 3 mM or $CaCl_2$ at 6 mM inhibited the enzyme activity about 50%.

Effect of the Cytosolic Fraction on Lysosomal Acid CEH Activity

The Acid CEH Activity was markedly inhibited by the addition of the cytosolic fraction. The inhibitory effect on Acid CEH activity was dependent on the concentration of the cytosolic protein, with the addition of about 50 μg of cytosolic protein causing approximately half maximal inhibition of Acid CEH activity (Fig. 3a).

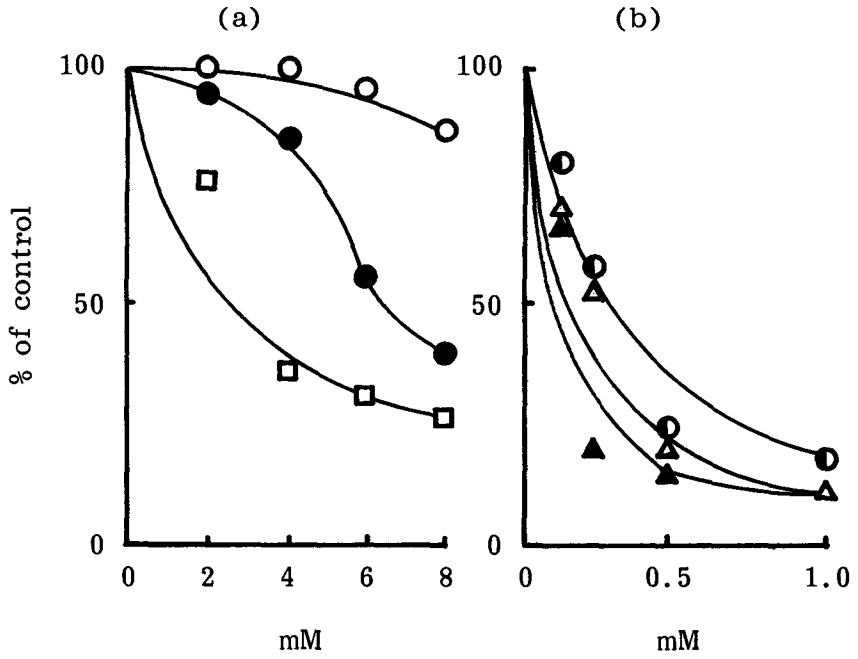

FIG. 2. Influence of various metals on Acid CEH activity. The 100% value is the activity under standard assay contitions. (a) • $CaCl_2$, ○ $MgCl_2$, □ $MnCl_2$; (b) △ $FeCl_2$, ▲ $FeCl_3$, ○ $ZnCl_2$.

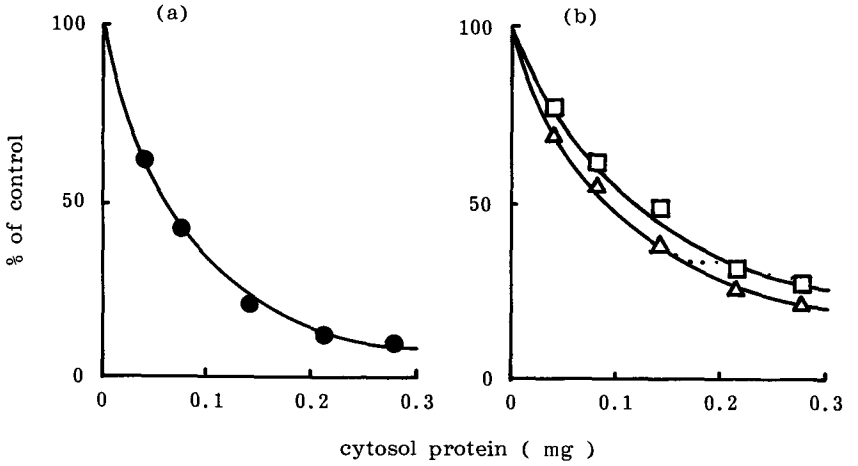

FIG. 3. Effect of the cytosolic fraction on Acid CEH activity. a) The substrate was dissolved in 20 μl saline containing 0.5% bovine serum albumin (•); b) The substrate was dissolved in 20 μl acetone (△) or 20 μl dimethylsulfoxide (□).

Since cholesteryl esters are generally insoluble in aqueous solution, we tested the inhibitory effect of the cytosolic fraction with 3 different modes of preparation of substrate: (a) 0.5%–albumin in saline solution (Fig. 3a), (b) acetone (Fig. 3b), and (c) dimethylsulfoxide (Fig. 3b). A similar inhibitory pattern was produced by the cytosolic fraction with all 3 ways of adding the substrate to the enzyme.

The kinetics of inhibition of Acid CEH, given in Figure 4a as Lineweaver-Burk plots, showed that the cytosolic protein inhibited Acid CEH competitively. The apparent Km value was 11.1 μM. Moreover, the inhibitor became more effective with incubation time (Fig. 4b).

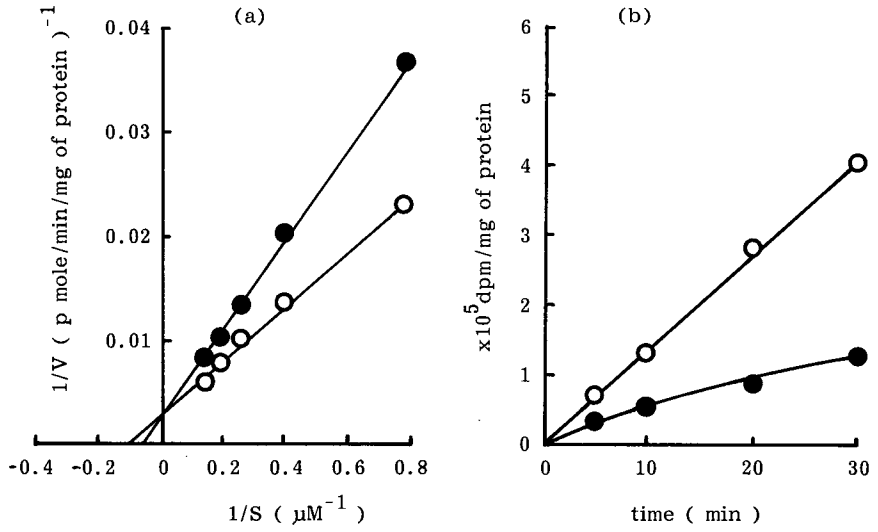

FIG. 4. Inhibitory effect of the cytosolic fraction on Acid CEH. a) Lineweaver-Burk plots of cholesteryl ester hydrolase in the presence (•) or absence (○) of the cytosolic protein (45 μg). b) Time course of the rate of hydrolysis in the presence (•) and absence (○) of the cytosolic protein (125 μg). Standard assay conditions were used as described in Materials and Methods.

Inhibitory Properties of Cytosolic Fraction on Acid CEH Activity

Table 1 shows the effect of various pretreatments of the cytosolic fraction on its capacity to inhibit Acid CEH activity. Boiling the cytosolic fraction for 5 min abolished most of the inhibitory effect. The inhibitory effect also was partly destroyed in cytosolic fractions pretreated with trypsin or chymotrypsin, while the inhibitory activity of cytosol was not influenced by steapsin and N-ethylmaleimide.

On the other hand, cytosol dialyzed overnight against 0.01 M Tris-HCl buffer (pH 7.4) or, exposed to repeated freezing-thawing, completely retained the inhibitory activity. After the cytosolic fraction was kept at pH 5.0 for 2 hr for the elimination of the lipid-soluble phosphate (14), 70% of its inhibitory activity remained.

Effect of Cytosolic Fraction on Other Enzymes

The cytosolic fraction did not inhibit 3 other lysosomal enzymes: cathepsin D, acid phosphatase and β-glucuronidase (data not shown). Furthermore, no inhibition of neutral CEH was observed (data not shown). The same concentrations of the cytosolic fractions were used in these studies as in Figure 3.

Tissue Distribution of Acid CEH and the Cytosolic Inhibitor

Acid CEH was present in all tissues tested, and high activity was observed in the spleen, lung, liver and heart. The cytosolic inhibitor also was present in various other tissues. Using the cytosolic protein concentration necessary for 50% inhibition of lysosomal Acid CEH activity in the liver as a standard, the concentration in other tissues would indicate inhibitory strength of cytosol to be especially high in the brain and heart, followed by the liver, kidney and lung (Fig. 5).

Identification of the Cytosolic Inhibitor

The pH 5.1 treated liver cytosol was fractionated by Sephacryl S-200 gel filtration. The eluate was monitored by absorption at 280 nm, and the inhibitory activities on Acid CEH and thiol proteinase (cathepsin B) were examined in each fraction. The Acid CEH inhibitory activity eluted in a broad peak (Fig. 6). Furthermore, the inhibitory activity for Acid CEH was distinguished in elution position from the inhibitory activity for thiol proteinase.

DISCUSSION

An inhibitor of lysosomal Acid CEH in vitro was found in the cytosolic fraction of liver and other tissues. The inhibitory strength was highest in brain and heart.

The cytosolic inhibitor was non-dialyzable, and was destroyed by heat treatment and partly destroyed by trypsin or chymotrypsin digestion. These results strongly suggest that

TABLE 1

Inhibitory Properties of Cytosol on Acid CEH Activity

Treatment	Acid CEH activity ($x10^5$ dpm/mg of protein/30 min)	Relative per cent
none	4.24	100.0
native cytosol	1.12	26.4
(a) dialyzed cytosol	1.21	28.5
(b) boiled cytosol	3.50	82.5
(c) freezing and thawing	1.17	27.6
(d) trypsin	2.91	68.5
(e) trypsin + trypsin inhibitor	1.38	32.5
(f) chymotrypsin	2.98	70.3
(g) steapsin	1.28	30.2
(h) N-ethylmaleimide	1.29	30.4
(i) pH 5.0 treatment	1.69	39.9

Assay conditions are given in Figure 3. (a) The cytosol was dialyzed overnight against 0.01 M Tris-HCl buffer (pH 7.4); (b) Boiled for 5 min and centrifuged at 3,000 rpm for 20 min; (c) Freezed and thawed 2 times; (d) Treated with trypsin (0.2%) for 15 min at 37 C, and then trypsin inhibitor was added (1.0%) before assay; (e) Treated with a mixture of trypsin inhibitor and trypsin; (f) Treated with chymotrypsin (0.2%); (g) Treated with steapsin (0.1%) for 15 min at 37 C; (h) N-ethylmaleimide was added to the cytosol (10 mM), after incubation at 37 C for 5 min, the mixture was dialyzed; (i) Adjusted to pH 5.0 with acetic acid and centrifuged at 10,000 rpm for 15 min.

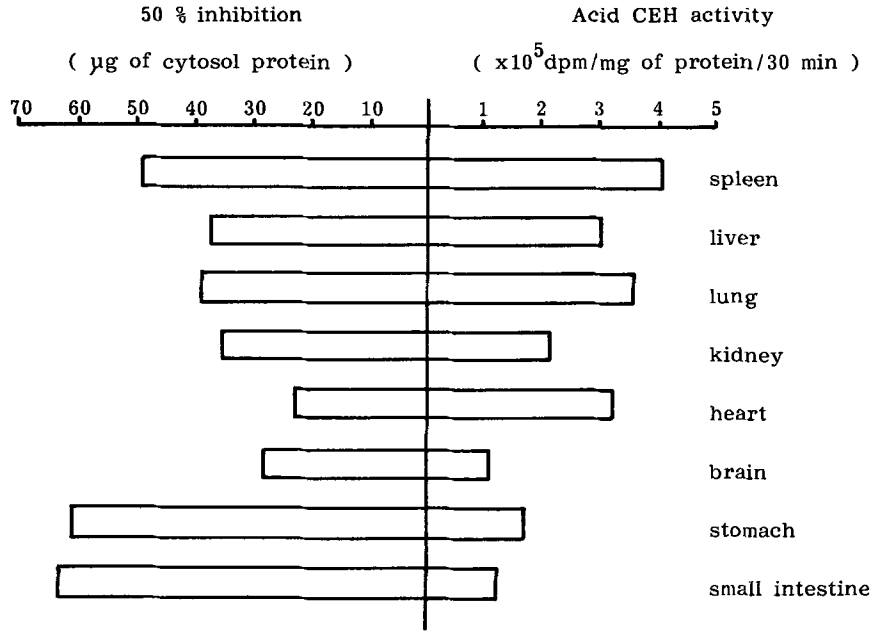

FIG. 5. Tissue distribution of Acid CEH activity and the cytosolic inhibitor. Standard assay conditions were used as described in Materials and Methods.

the lysosomal Acid CEH inhibitor present in cytosol is protein in nature or is attacted to cytosolic protein.

It has been reported that many kinds of protein inhibitors are present in various tissues and serum. Gorin et al. (15) indicated that the inhibitor of acid lipase in cultured fibroblasts was present in Chon fraction IV of human and calf serums. In addition, Kubo et al. (16) recognized that human plasma lipoprotein A-I and A-II inhibited the hydrolysis of triglyceride catalyzed by hepatic triglyceride lipase. These results show that the inhibitory effect of serum on lipase activity is due to a serum protein

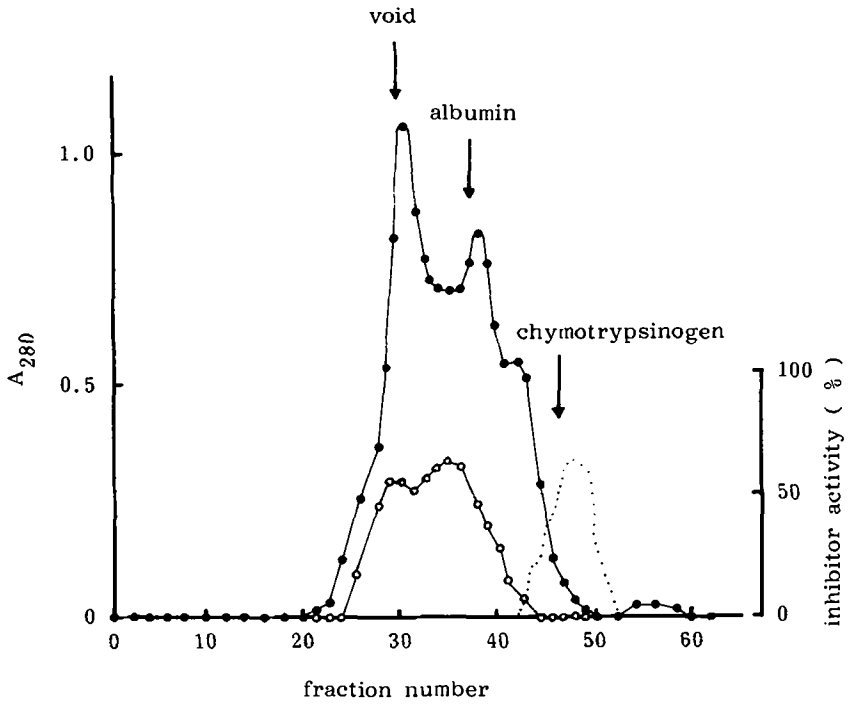

FIG. 6. Gel filtration of pH 5.1 treated liver cytosol on Sephacryl S-200. The column was equilibrated with a 0.1 M NaCl, 0.01 M phosphate buffer, pH 7.5, at 4 C. •—•, absorbance at 280 nm; o—o, inhibitory activity on Acid CEH; ------, inhibitory activity on cathepsin B. The column was calibrated with the following markers: Blue Dextran 2,000; albumin (mol. wt. 66,000); chymotrypsinogen (21,500).

component. On the other hand, the results presented here show that Acid CEH activity is strongly inhibited by a liver protein component. However, it is not obvious whether liver inhibitor and serum inhibitor are the same, because the liver inhibitor has not been purified.

A thiol proteinase inhibitor in rat liver cytosol has been purified and characterized (8,17). This inhibitor is present in the cytosolic fraction of all rat tissues. It is very heat stable and loses inhibitory activity when treated with sulfhydryl reagent such as Ellman reagent or N-ethylmaleimide. However, the present Acid CEH inhibitor is heat labile, and its inhibitory activity was not affected by treatment with these sulfhydryl reagents. Furthermore, gel filtration profile on Sephacryl S-200 of the present cytosolic inhibitor was different from that of a thiol proteinase inhibitor. The cytosolic inhibitor seems to correspond to a protein of approximately Mr = 80,000 and, in addition, a higher molecular weight protein that eluted in the void volume from a Sephacryl S-200 column. But, it is not clear at the present whether more than one protein may be responsible for the inhibitory activity.

Neiderhiser et al. (18) has also reported that a protein present in the cytosol of guinea pig gall bladder mucosa competitively inhibited the activity of mitochondrial Acid CEH. But, it is not clear at the present whether the rat liver cytosolic inhibitor of Acid CEH is identical to the cytosolic inhibitor in guinea pig gall bladder.

Lysosomal Acid CEH in involved in the catabolism of cholesteryl esters introduced into the liver as lipoproteins (19). This enzyme was inhibited by the cytosolic protein. The findings suggest that the cytosolic inhibitor may be related to the regulation of utilization of cholesteryl esters in the cell. However, any physiological significance of the presence of an active Acid CEH inhibitor in the cytosol remains to be established.

REFERENCES

1. Stoffel, W., and Greten, H. (1967) Hoppe-Seylers Z. Physiol. Chem. 348, 1145-1150.
2. Brown, W.J., and Sgoutas, D.S. (1980) Biochim. Biophys. Acta. 617, 305-317.
3. Stokke, K.T. (1972) Biochim. Biophys. Acta.

270, 156-166.
4. Dousset, J.C.; Dousset, N.; Foglietti, M.J., and Douste, B.L. (1981) Biochim. Biophys. Acta. 664, 273-277.
5. Peters, T.J.; Takano, T., and De Duve, C. (1973) in Atherosclerosis Initiating Factor, Ciba Foundation Symposium 12 (Porter, R., and Knight, J., eds.), pp. 197-222, Elsevier, New York.
6. Takano, T., and Imanaka, T. (1978) Acta. Histochem. Cytochem. 11, 323-336.
7. Brecher, P.; Chobanian, J.; Small, D.M., and Chobanian, A.V. (1976) J. Lipid Res. 17, 239-247.
8. Hirado, M.; Iwata, D.; Niinobe, M., and Fuji, S. (1981) Biochim. Biophys. Acta. 668, 21-27.
9. Lowry, O.H.; Rosebrough, N.J.; Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
10. Lenney, J.F.; Tolan, J.R.; Suga, W.J., and Lee, A.G. (1979) Eur. J. Biochem. 101, 153-161.
11. Gianetto, R., and De Duve, C. (1955) Biochemistry 59, 433-455.
12. Shibko, S.; Aram, V., and Tappel, A. (1965) Biochem. J. 95, 731-741.
13. Shinomiya, M.; Matsuoka, N.; Shirai, K.; Saito, Y., and Kumagai, A. (1979) Atherosclerosis 33, 343-350.
14. Murphy, D.J.; and Mavis, R.D. (1981) J. Biol. Chem. 256, 10464-10468.
15. Gorin, E.; Gonen, H., and Dickbuch, S. (1982) Biochem. J. 204, 221-227.
16. Kubo, M.; Matsuzawa, Y.; Yokoyama, S.; Tajima, S.; Ishikawa, K.; Yamamoto, A., and Tarui, S. (1981) Biochem. Biophys. Res. Communs. 100, 261-266.
17. Jarvinen, M. (1979) FEBS Lett. 108, 461-464.
18. Neiderhiser, D.H. (1982) Biochem. Biophys. Res. Communs. 105, 328-333.
19. Goldstein, J.L., and Brown, M.S. (1977) Annu. Rev. Biochem. 46, 897-930.

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Homeostasis of Mucosal Cholesterol in the Small Intestine of the Rat

A. BLUMER, S.M. WATT* and W.J. SIMMONDS, *Department of Physiology, University of Western Australia, Nedlands, Western Australia, 6009*

ABSTRACT

This study was undertaken to investigate the capacity of the intestinal mucosa to maintain a constant cholesterol content under conditions where mucosal uptake or cholesterol transport into the lymph were manipulated. Two series of bile-diverted unanaesthetised rats were infused intraduodenally with saline, triolein emulsified with Pluronic F68, or taurocholate with or without added tomatine. Pluronic F68 is a nontoxic detergent which promotes mucosal uptake of polar lipids but not cholesterol. Tomatine is a cholesterol-binding saponin. One series of rats was used for measuring mucosal cholesterol content, DNA and protein after the test infusions. A second series of rats had the thoracic lymph duct cannulated but otherwise remained the same as the first series. The second series was used for measuring the effect of the different infusions on mass cholesterol output into lymph. Mucosal cholesterol content of rats that were not fed decreased with bile-diversion and was restored with taurocholate infusion. This suggested a contribution of luminal cholesterol to the mucosal cholesterol pool. However, evidence for a contribution from the lumen was provided by only one of two groups of rats given infusions which did not promote mucosal uptake of cholesterol. First, addition of tomatine to the taurocholate infusate prevented both the increase in lymph output of cholesterol and the increased mucosal cholesterol content shown in rats given taurocholate alone. Second, in another group of rats in which mucosal uptake of cholesterol was prevented, i.e. in rats given Pluronic F68-triolein emulsions, the increased fat absorption was accompanied by a marked increase in cholesterol output into lymph without a concomitant decrease in mucosal cholesterol content. These results would be consistent with increased mucosal synthesis of cholesterol as a possible source of endogenous cholesterol absorbed into lymph.

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INTRODUCTION

In most cells there is a continuous turnover of cholesterol. A fairly steady cell content implies mutual adjustment of uptake, synthesis and removal by homeostatic mechanisms (1). In the liver and intestine, which play the major role in overall homeostasis of body cholesterol, turnover is rapid and large relative to tissue content. The cells in tissues of both organs are polarized with respect to gain and loss, in the intestine more strongly than in the liver.

The canalicular side of the hepatocyte is a one-way channel for loss of cholesterol, as biliary cholesterol and as newly synthesized bile acids. However, there is both gain and loss of cholesterol from the sinusoidal side—by uptake of soluble lipoproteins and remnant particles and by export of nascent lipoproteins. This two-way traffic is difficult to quantitate or manipulate in relating the cholesterol content of hepatocytes to altered gains or losses.

Cholesterol uptake and removal are more strongly polarized in the enterocyte. Cholesterol is taken up from the intestinal lumen through the brush border and removed basolaterally into lymph as lipoproteins, mainly chylomicrons and very low density lipoproteins (VLDL). To some extent, steady-state uptake and loss can be varied independently. Changes in cholesterol content then should reflect concomitant adjustments in synthesis. A considerable amount of work has been done on the role of bile salts and luminal cholesterol in controlling mucosal synthesis, measured isotopically (2,3). However, there is relatively little information on mass balance of cholesterol gains and losses by the mucosa.

In the experiments presented here, two aspects of mucosal cholesterol balance have been manipulated in unanaesthetized rats. Uptake from the lumen has been virtually abolished by bile fistula and restored by replacing bile salts. Export of cholesterol to the lymph has been stimulated, in the absence of cholesterol uptake, by giving fat emulsified with a non-ionic detergent to bile fistula rats. The non-ionic detergent, Pluronic F68, substitutes for bile acids in triglyceride absorption but has no effect on cholesterol absorption (4).

The fasting mucosal content decreased with bile fistula and was restored by replacing bile salts without biliary cholesterol. These changes were accompanied by decreased and increased output, respectively, of cholesterol in lymph. Such results suggested a partial dependence of mucosal cholesterol content on cholesterol

*To whom correspondence should be addressed.

supply from the lumen. However, in the absence of cholesterol uptake, fat absorption increased cholesterol transport into the lymph several fold without a concomitant decrease in mucosal cholesterol content. The cholesterol exported in 8 hr was comparable with the mucosal content in a bile fistula rat. This is consistent with a homeostatic role of increased cholesterol synthesis.

MATERIALS AND METHODS

Experimental Design

Male rats of an inbred Wistar strain weighing 150-200 g were used in the experiments. There were two series of rats. In Series A, 35 rats were divided into 6 groups. Each rat was infused at a constant rate (1.5 ml/hr) through an intraduodenal cannula. All rats except those in group 1 were bile-diverted. In groups 1-3 the composition of the infusate remained unchanged for 48 hr. Groups 4-6 were given a basic glucose saline infusate for 40 hr, followed by an 8-hr test infusion delivered at the same flow rate. At the end of 48 hr the rats were killed and the mucosal content of cholesterol, protein and DNA in the small intestine was determined.

In Series B the cholesterol output in lymph was measured in 17 rats with cannulae in the abdominal thoracic duct. They were divided into the same 6 groups and treated in the same way as in Series A except that the volume of the intestinal infusate was doubled (3 ml/hr) to maintain hydration.

Treatment of the 6 groups is summarized in Table 1.

Surgical Procedures

Rats not fed overnight had one or more cannulae inserted under ether anaesthesia as described previously (5). All rats had an intraduodenal cannula through which fluid was infused after surgery. In Series A and B, in all rats except those in group 1, bile was drained through a cannula inserted just distal to the bifurcation of the bile duct. This allowed pancreatic juice to enter the duodenum normally. In Series B the rats' lymph was collected through a cannula inserted into the abdominal thoracic lymph duct just above the cisterna coli. After surgery the rats were transferred to restraint cages maintained at 30 C where they were infused with a basic glucose saline infusate of 10 mM glucose, 4 mM KCl and 143 mM NaCl.

Infusates

Test infusates for groups 4-6 were made up in phosphate-buffered saline (6.75 mM Na_2HPO_4, 16.5 mM $Na\,H_2PO_4$, 115 mM NaCl, 5 mM KCl, pH 6.4) containing 10 mM glucose and 1 mM $CaCl_2$. The triolein emulsion was prepared by adding the required amount of Pluronic F68 (BASF, Wyandotte Corp., Michigan) mixed in a small volume of phosphate buffer to the triolein. This mixture was sonicated with gradual additions of more warm buffer until the required volume of stable emulsion was obtained.

Taurocholate, 10 mM, was added for groups 3, 4 and 5. In group 6 tomatine (U.S. Biochemical Corp., Cleveland, Ohio) 0.5 mg/ml was added to the taurocholate infusate.

Collection of Samples and Analyses

At the end of the 8-hr infusion rats were anaesthetised with ether and the small intestine from the Ligament of Treitz to the lower ileum was rapidly and carefully excised before cardiac puncture. Upper and lower halves were then washed through with chilled saline. Preparation of mucosal homogenates was carried

TABLE 1

Summary of Treatment of 6 Groups of Rats (Series B) During Measurement of Cholesterol Output in Lymph

Group number	Bile diverted	48-hour infusion
1	–	Glucose-saline for 48 hr
2	+	Glucose-saline for 48 hr
3	+	Glucose-saline + 10 mM NaTC for 48 hr
4	+	Glucose-saline for 40 hr, glucose-saline + 10 mM NaTC for 8 hr
5	+	Glucose-saline for 40 hr, glucose-saline + 10 mM NaTC + 0.5 mg/ml Tomatine for 8 hr
6	+	Glucose-saline for 40 hr, Triolein + Pluronic F68 for 8 hr

out at 4 C. The mucosal scrapings were homogenized by sonication in 20 ml saline per half intestine. Aliquots taken for cholesterol assay were extracted by the method of Blankenhorn and Ahrens (6) and saponified (7). Cholesterol was determined by the method of Zlatkis and Zak (8). Appropriate reagent blanks and standards were extracted, saponified and assayed in the same way.

Hourly lymph samples were collected over ice into heparinized tubes and extracted by the method of Blankenhorn and Ahrens (6). Aliquots of the lipid extracts were taken for total cholesterol and for thin layer chromatography (TLC) on 25 mm layers of Silica gel G (Merck, Darmstadt, Germany) and plates prewashed in chloroform-methanol (2:1, v/v). Plates were developed in hexane:diethyl ether:glacial acetic acid (70:30:2, v/v/v) to separate the neutral lipids. Free cholesterol and esterified cholesterol were identified against plated reference standards and eluted from the plate with chloroform:methanol (2:1, v/v), which was evaporated off before saponification and determination of cholesterol as for mucosal homogenate extracts.

Protein was determined by the Lowry method (9). DNA was measured by a modification of the method of Burton (10). Two ml aliquots from the 20 ml mucosal homogenate were washed twice with 5 ml of ice cold 0.3 M perchloric acid (PCA) by centrifuging (IEC-PR 6000) at 2500 rpm for 5 min. The pellet was then resuspended in 8 ml of ice cold 0.8 M PCA and incubated at 70 C for 45 min with shaking. During incubation the samples were well-mixed every 10 min. The samples were made up to 10 ml with a 0.8 M PCA and again centrifuged at 2500 rpm for 5 min. Two ml of color reagent (100 ml of 1.5% diphenylamine in glacial acetic acid, 0.5 mls 2% acetaldehyde in H_2O and 1.5 ml conc. H_2SO_4) was added to duplicate lml aliquots of each supernatant.

The samples, together with blanks and standards, were then left for 16-19 hr at 37 C and read at 600 nm (Varian Techtron spectrophotometer G35).

Materials

Cholesterol and triolein (TO) were purchased as high purity grade (>99% pure) from the Nu-chek-Prep Inc., Elysian, Minnesota and used as supplied. Sodium taurocholate was prepared by the method of Lack et al. (11) and moved as one spot on TLC plates developed in propionic acid-isoamylacetate-water-n-propranalol, 15:20:5:10 (v/v/v/v). O-phthalaldehyde was from Sigma, St. Louis, Missouri. All reagents and solvents were of analytical grade except ethanol, which was redistilled.

Statistical Analysis

The results are expressed as mean values and standard error of the means. Student's t-test was used to determine the significance of the differences.

RESULTS

Mucosal Cholesterol

The results in 6 groups of rats in Series A, 5 groups with bile fistula and one group with bile ducts intact, are summarized in Table 2.

Bile fistula (40 hr) decreased the fasting cholesterol content of the mucosa, per mg DNA, by about 20% (compare groups 1 and 2, $p < 0.01$). The decrease was prevented by replacing bile salts as sodium taurocholate (TC), 30 micromoles per hr, from the time of operation (group 3).

Duodenal perfusion of TC for 8 hr also raised the mucosal content in bile fistula rats to values found in rats with bile ducts intact (compare groups 4 and 2, $p < 0.001$, and groups 4 and 1, ns). The effect of TC was abolished by adding tomatine, 0.5 mg per ml, to the perfusate (group 5). Tomatine precipitates cholesterol but not bile salts from micellar solution.

An increased loss of cholesterol from mucosa to lymph, without an equivalent compensatory uptake from the lumen, was produced by duodenal perfusion of bile fistula rats with triolein in the non-ionic hydrophilic detergent, Pluronic F68 (group 6). Lymphatic cholesterol output was considerably increased, see below, but the mucosal cholesterol content remained the same as in saline-perfused bile-fistula rats (compare groups 2 and 6).

The protein content of the mucosa per mg DNA was significantly decreased by bile fistula (compare groups 1 and 2, $p < 0.01$) but did not vary significantly among the differently treated bile-fistula groups.

Lymph Cholesterol Output

Only the mucosal content was studied in the above 6 groups, comprising 30 rats (Series A). Mucosal losses to the lymph were measured in another 17 rats with lymph fistulae (Series B). The effects of the various procedures on cholesterol output in the lymph are shown in Figure 1. Three of the groups were presumably in a fairly steady state of mucosal cholesterol turnover (Fig. 1A). Bile fistula rats perfused only with saline had a low, steady output of lymphatic cholesterol. This was increased several fold by steady infusion of TC and probably still more

TABLE 2

Effect of Bile Diversion, Taurocholate and Lipid Infusion on Cholesterol Content of Intestinal Mucosa

Procedure[a]	Group	Number of rats	Cholesterol (μg/mg DNA			Protein (mg/mg DNA)		
			Total	Upper	Lower	Total	Upper	Lower
Sham operated[b]	1	(7)	357[c] ±17.2 **[d]	350 ±21.6 *[e]	366 ±22.1 *	16.7 ±0.82 **	17.4[f] ±1.12	16.1 ±1.11 *
Bile fistula	2	(6)	284 ±10.4	282 ±12.6	289 ±17.0	13.8 ±0.28	14.9 ±0.75	13.0 ±0.26
Bile fistula + TC (48 hr)	3	(5)	334 ±26.9	339 ±16.8 *	331 ±38.8	14.7 ±0.83	15.2 ±0.91	14.3 ±0.93
Bile fistula + TC (8 hr)	4	(7)	356 ±16.3 **	368 ±31.1 *	349 ±15.6 *	14.5 ±0.50	14.9 ±0.64	14.2 ±0.49
Bile fistula + TC + tomatine	5	(5)	243 ±15.5	237 ±16.5	248 ±17.2	15.4 ±1.05	15.4 ±0.92	15.4 ±1.19
Bile fistula + triolein + Pluronic F68	6	(5)	281 ±22.4	284 ±25.6	277 ±20.9	14.4 ±0.48	15.1 ±0.48	13.7 ±0.51

[a]All rats given glucose-saline infusion for the 40 hrs preceding the 8-hr test infusion except for group 3 where rats were infused with 10 mM TC continuously for 48 hr.

[b]Rats in group 1 had intraduodenal cannulae for infusion.

[c]Values represent mean ±SEM for the number of rats shown.

[d]**$p < 0.01$ when compared with group 2.

[e]*$p < 0.05$ when compared with group 2.

[f]Mean values given without an asterisk were not significantly different from mean values for rats in group 2 ($p > 0.05$).

in rats with bile ducts intact, although the numbers of lymph fistula rats are too small for statistical evaluation. The effects in bile fistula rats of changing the composition of the perfusate after 40 hr perfusion of saline alone are shown in Figure 1B. Steady perfusion of TC for 8 hr increased cholesterol output at first rapidly and then more slowly to a level somewhat higher than in rats perfused with TC for more than 40 hr. When tomatine was added to the TC perfusate the increase was abolished. This suggested that the increased cholesterol output with TC alone was derived from the lumen. An increased output of cholesterol comparable to that with TC was obtained by infusing triolein emulsified in Pluronic F68. Under these circumstances no cholesterol was absorbed from the lumen, the increase being derived from the cells during assembly of chylomicrons containing absorbed triglyceride.

In Figure 2, the lymphatic output of cholesterol during the final 8 hr perfusion in the second series of experiments (Series B), is compared with the mucosal cholesterol content of the whole small intestine in the first series (Series A). The 8-hr turnover was considerable, ranging from about 20% of mucosal content in bile fistula rats to over 60% in rats with bile ducts intact.

DISCUSSION

The object of these experiments was to measure the effect on mucosal cholesterol content of altering gain from the lumen or loss to the lymph. Exogenous cholesterol was excluded. No food was given throughout the experiments, except for glucose (approx. 0.4 kcal/day) in the perfusate. Mucosal cholesterol was measured 48 hr after operation and placement in a restraint cage.

Bile fistula deprives the animal of bile salts which are essential for the uptake of cholesterol from the lumen. Under these conditions the output of cholesterol in the lymph was about one-quarter of that in animals with bile ducts intact. This diminution (by about 300 μg per hr) presumably reflects the absence of turnover

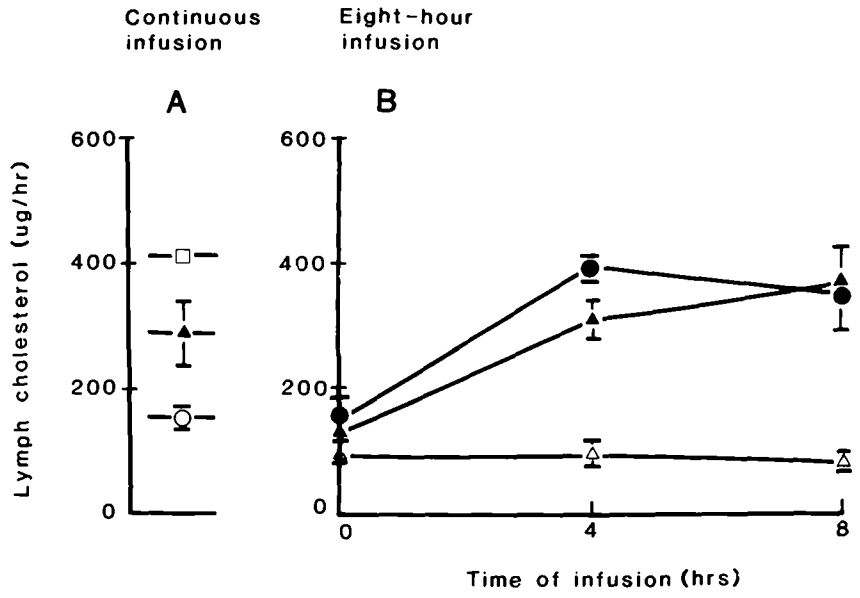

FIG. 1. Lymph output of cholesterol showing (A) steady values obtained during continuous infusions and (B) output when the infusates were given for 8 hr commencing 40 hr post-operatively. Lymph fistula rats were infused intraduodenally at 3 ml/hr. Except for one group, group 1 (□), all rats were bile-diverted. Two rats given 10 mM NaTC continuously after surgery (group 3) were used to measure steady lymph output rate (left panel, ▲—▲) and 3 rats (group 4) were given the same infusate for 8 hr (right panel, ▲——▲); 3 rats (group 2) were given saline (○); 3 rats (group 5) were infused with tomatine 0.5 mg/ml in 10 mM NaTC for 8 hr (△——△) and 2 rats (group 6) were given TO emulsified with Pluronic F68 for 8 hr (●——●). For comparison, the steady rate cholesterol output for a bile intact rat given saline is shown (□). Values are μg/hr, means ±S.E.M. for the number of rats shown except for group 6, where the mean and range of the two values is given. Further experimental details in Materials and Methods.

from uptake of luminal cholesterol. There still was a substantial output of cholesterol in the lymph (about 100 μg per hr, equivalent to about 50% of mucosal content per day). It cannot be assumed, however, that this cholesterol or even the major part of it reflects turnover of cholesterol synthesized in the mucosa. On the one hand, some of the cholesterol in the lymph is derived from passage of lipoproteins from plasma to tissue fluids. Uptake of lipoprotein cholesterol may occur via the lipoprotein binding sites now reported in isolated intestinal mucosal cells (12). On the other hand, some of the cholesterol synthesized in the mucosa may be lost into the lumen (13).

Mucosal cholesterol per unit DNA was significantly decreased by bile fistula. Thus, even if mucosal synthesis (2) or transfer from plasma was increased in the absence of bile, this did not fully compensate for the absence of uptake from the lumen.

The lowered mucosal content in bile fistula rats was not further decreased when loss of cholesterol by export in chylomicrons was increased during fat absorption. A non-ionic detergent, Pluronic F68, was substituted for the missing bile salts. This detergent was as efficient as bile salts in promoting fat absorption in bile fistula animals but was unable to mediate cholesterol absorption (4). With the dose of fat given, the lymphatic output of cholesterol exceeded that in fasting bile fistula rats by a total of 1700 μg in 8 hr, equivalent to 40% of the total mucosal cholesterol. Since uptake of cholesterol was negligible, there must have been a considerable increase in mucosal synthesis or uptake from plasma associated with chylomicron production.

These results can be compared with those when taurocholate was infused in the absence of biliary lipid. The increase in lymphatic output of cholesterol was similar, but the fat output increased only slightly. The mucosal content of cholesterol was increased to values near those in fasting rats with bile ducts intact. This suggested a considerable uptake of non-biliary

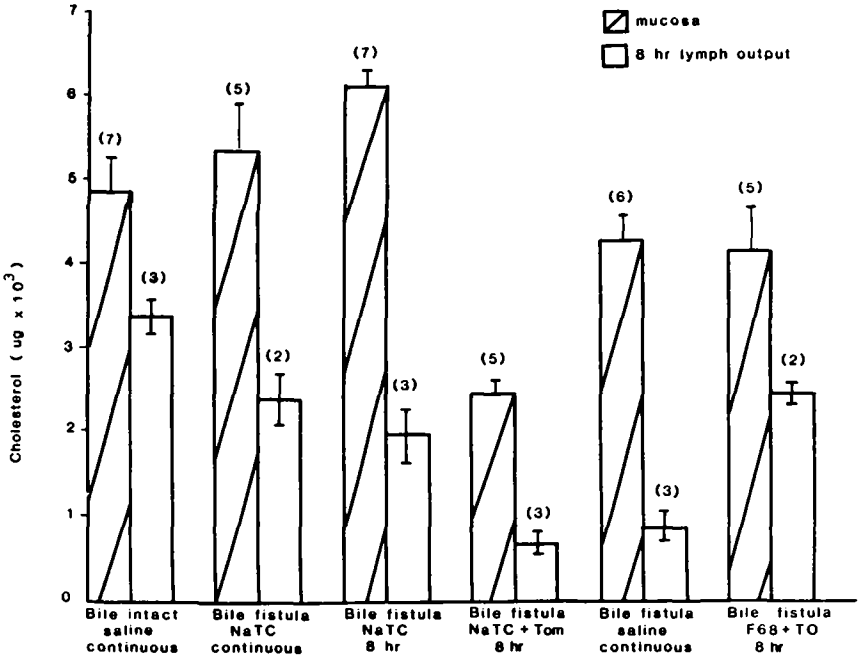

FIG. 2. Comparison of total cholesterol content of the intestinal mucosa after 8 hr infusions and total cholesterol collected in lymph during 8 hr infusions with the same solutions. Cholesterol values represent the cholesterol extracted from the mucosal scrapings of the total small intestine (μg/rat) or from lymph (μg/8 hr). Rats used for mucosal values differed from those used for lymph values in having intact lymph flow. Values are means ±S.E.M. for the number of rats shown in parentheses except for those lymph values where only 2 rats were used. For these 2 groups error bars indicate the range between the two values. Experimental details in Methods.

cholesterol from the lumen, mediated by taurocholate even in the absence of biliary cholesterol. The effects of adding tomatine to the taurocholate perfusate were consistent with this but were by no means conclusive. Tomatine has been claimed to precipiate cholesterol but not bile salts from micellar solution in the lumen (14). Tomatine abolished the increases both in lymphatic output of cholesterol and in mucosal cholesterol content found with taurocholate alone. However, the biological effects of tomatine are uncertain. With tomatine plus taurocholate the mucosal cholesterol content per unit DNA was lower than with saline alone. It is possible that tomatine removed cholesterol from mucosal cells. Also, the lumen appeared to contain more fluid and mucus than normal. This suggests that tomatine may have direct effects on mucosal cell function.

The present experiments are regarded only as exploratory. For example, prolonged fasting may affect homeostatic adjustments by decreasing mucosal synthesis of cholesterol (15). Total mucosal cholesterol may not reflect changes in proliferating crypt cells or villus absorptive cells or in subcellular fractions such as brush borders or intracellular membranes. Nevertheless, the findings raise questions on the relationship of changes in the mucosal cholesterol pool to adjustments in uptake, synthesis and export which warrant further study.

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REFERENCES

1. Small, D.M. (1977) New Engl. J. Med. 297, 873-877.
2. Dietschy, J.M. (1968) J. Clin. Invest. 47, 286-300.
3. Peng, S.K.; Ho, K.J., and Taylor, C.B. (1974) Exp. Mol. Path. 21, 138-153.
4. Watt, S.M., and Simmonds, W.J. (1976) Clin. Exp. Pharmacol. Physiol. 3, 305-322.
5. Watt, S.M., and Simmonds, W.J. (1981) J. Lipid Res. 22, 157-165.
6. Blankenhorn, D.H., and Ahrens, E.H. Jr. (1955) J. Biol. Chem. 212, 69-81.
7. Abell, L.L.; Levy, B.B.; Brodie, B.B., and Ken-

dall, F.E. (1952) J. Biol. Chem. 195, 357-366.
8. Zlatkis, A., and Zak, B. (1969) Anal. Biochem. 29, 143-148.
9. Lowry, O.H.; Rosebrough, N.J.; Farr, A., and Randall, R.R. (1951) J. Biol. Chem. 193, 265-275.
10. Burton, K. (1956) Biochem. J. 62, 315-323.
11. Lack, L.; Dorrity, F.O. Jr.; Walker, T., and Singletary, G.D. (1973) J. Lipid Res. 14, 367-369.
12. Suzuki, N.; Fidge, N.; Nestel, P., and Yin, J. (1983) J. Lipid Res. 24, 253-264.
13. Wilson, J.D., and Reinke, R.T. (1968) J. Lipid Res. 9, 85-92.
14. Cayen, M.N. (1971) J. Lipid Res. 12, 482-490.
15. Cayen, M.N. (1969) Biochim. Biophys. Acta 187, 546-554.

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Serum Lipids and Fatty Acid Composition Of Tissues in Rats on Total Parenteral Nutrition (TPN)

F.M. MARTINS,[b,c] A. WENNBERG,[a] S. MEURLING,[b] R. KIHLBERG[a] and L. LINDMARK,[a,*] [a]*Vitrum Institute for Human Nutrition, KabiVitrum AB, Stockholm, and* [b]*Department of Pediatric Surgery, University Hospital, Uppsala, Sweden,* [c]*Present address: Department of Pediatric Surgery, S.4, Hospital D. Estefania, Lisbon, Portugal*

ABSTRACT

The modulating effect of energy and exogenous triglyceride supply on serum lipids and fatty acid composition of liver, tibialis anterior muscle, subcutaneous and peri-epididymal fat was assessed in rats using Total Parenteral Nutrition (TPN). Nutrients were infused continuously for 10 days through a central vein catheter. Four levels of fat were tested: No fat, Low fat (6% non-protein calories), Medium fat (30%) and High fat (60%), at 2 energy levels (270 kcal and 350 kcal/kg · day). An isonitrogenous supply was given to all groups as 0.9 g N/kg · day. At the highest level of fat intake (20.4 g/kg) at the high energy level triglycerides, cholesterol and phospholipids of serum were elevated. Biochemical signs of essential fatty acid (EFA) deficiency were observed as rising levels of eicosatrienoic acid and lowered concentrations of linoleic and arachidonic acid, mostly in the liver and the muscle in the No fat groups. EFA levels were reduced significantly in the No fat and Low fat groups and more pronounced at the high energy level. In conclusion, our study suggests that when energy intake is increased the EFA status becomes more critical. It seems evident that more EFA is required in absolute values when hypercaloric diets are given.
Lipids 19:728-737, 1984.

INTRODUCTION

Deficiency of linoleic acid (18:2ω6) and alpha-linolenic acid (18:3ω3) leads to well characterized syndromes, first described in the rat by Burr and Burr (1), and subsequently by Hansen et al. (2) studying orally fed children. EFA deficiency has been described in children after only one week of TPN with only glucose as energy substrate (3,4), and in adults on long-term TPN (5,6). Newborn individuals seem to be particularly susceptible to EFA deficiency, due to low fat reserves. Linoleic acid is the most important of the EFA's, the requirement for which still has not been precisely determined. An expert group within Food and Agricultural Organization/World Health Organization (FAO/WHO) (7) proposed that at least 3% of the energy requirements in adults, and 7% in pregnant and lactating women, should be met by linoleic acid. In children, Adam (8) proposed in 1958 that 4% of the energy should be supplied as linoleic acid, and today this value is accepted by most authors as covering children's daily requirements.

In mammals, fat deposits constitute the most important form of energy reserves, readily available in periods of energy shortage. Lipids, stored in adipose cells as triglycerides (TG), are submitted to a permanent flux of lipolysis and lipogenesis, modulated by genetic factors, nutritional status, hormonal milieu and substrate support. Adipose tissue, muscle and liver tissue are the most active tissues in the intermediate lipid metabolism. Using a rat model and TPN, this study was designed to investigate how the concentrations of fatty acids in the above mentioned tissues were affected by extreme situations of fat deprivation and fat overload, as well as by more balanced regimens, and also to investigate the influence of energy intake on lipid metabolism.

In a separate publication, we have reported the results from this study regarding body growth, nitrogen balance and body composition (9).

*To whom correspondence should be addressed at P.O. Box 12170, S-102 24 Stockholm, Sweden.

MATERIALS AND METHODS

Animals and Pre-treatment

Sixty-four male Sprague Dawley rats, 37-38 days old (Anticimex AB, Stockholm, Sweden), were divided into 8 groups and housed in individual cages. The animals were kept at an environmental temperature of 21 C, on a dark-light 12-hr cycle from the day they arrived at the laboratory. They were fed ad libitum a complete seimi-synthetic 19% casein diet supplemented with 0.3% L-methionine. During one week, the rats were adapted to a harness, especially designed to protect a central vein catheter. Under general anesthesia, a silicone catheter was then implanted in the superior caval vein, through the internal jugular vein and tunnelized to the back of the rat and through a hole in the harness. In the next six days, the

rats recovered, while being fed ad libitum a nitrogen amino acid diet. The body weights of the rats when the infusion procedure started were about 200 g. For further details of the diets see Martins et al. (9).

Study Design

Two different energy levels, 270 kcal and 350 kcal/kg • day, and 4 fat/glucose energy ratios were tested: "No fat" (N, 0% fat), "Low fat" (L, 6% fat), "Medium fat" (M, 30% fat) and "High fat" (H, 60% fat). Each group included 7-9 animals.

Nutrients and Infusion Procedure

The nutrients were administered continuously for 10 days, through a central vein catheter, at a constant rate. Every day, new infusion solutions were mixed, aseptically, under laminar air flow (Table 1). Amino acids were administered to all rats at a constant dose of 0.9 g N/kg day, and provided as a complete amino acid solution (Vamin® N, KabiVitrum AB, Stockholm). Fat was administered as Intralipid® 20% (KabiVitrum AB, Stockholm) (Table 2). Carbohydrate was administered as glucose 50% in water. The infused volume was adjusted to 330 ml/kg • day, using distilled sterile water. Vitamins and minerals also were added, in amounts adapted to the daily requirements of the rats. During the infusion procedure, the animals were housed in metabolic cages that allowed them to move freely.

Tested Parameters

After completing a 10-day period on TPN, two hr after infusion ended, the rats were anesthetized with sodium pentobarbital injected intraperitoneally and were killed by exsanguination through the heart. Liver and samples from epididymal and sub-cutaneous fat were taken, immediately frozen in liquid nitrogen and kept at −70 C until analyzed. Tibialis anterior muscle (right) also was excised and equally frozen and kept at −70 C.

Serum lipid classes. Serum lipids were analyzed during infusion (day 2). Blood samples, obtained from the tail, were analyzed for serum triglycerides (TG) according to Fletcher et al. (10), for serum total cholesterol according to Zlatkis et al. (11), for phospholipid (PL) according to Svennerholm et al. (12) and for cholesterol esters and free fatty acids (FFA) according to Richmond (13).

Fatty acid profiles. Fatty acid composition of the liver, tibialis anterior muscle, epididymal and sub-cutaneous fat pads were determined by means of gas liquid chromatography (GLC) (14), slightly modified in our laboratory.

Statistical methods. The data, presented as mean values ± standard error of the means (SEM), were compared using Student's t-test for unpaired samples, and the differences considered significant at a probability level less than 0.05.

RESULTS

The outcome of this study can be divided into 2 parts, serum lipids, and fatty acid composition of some tissues. The results are presented in Tables 3-7 and Figures 1-2.

Serum Lipids

The serum triglycerides during TPN (Table 3)

TABLE 1

Amounts of Energy and Nutrients Infused Daily as TPN During the Infusion Period (expressed per kg bodyweight and day)*

Total energy kcal	270	270	270	270	350	350	350	350
Non-protein energy supply kcal	240	240	240	240	320	320	320	320
Groups	N	L	M	H	N	L	M	H
Fat/carbohydrate energy ratio (%)	0/100	6/94	30/70	60/40	0/100	6/94	30/70	60/40
Component	Given amounts (nominal values)							
Nitrogen g	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
Glucose g	64	59	42	21	84	79	56	29
Triglycerides g	0	1.5	7.7	15.3	0	2.0	10.2	20.4
Egg yolk phospholipids g	0	0.1	0.5	0.9	0	0.1	0.6	1.2
Glycerol g	0	0.2	0.9	1.8	0	0.2	1.2	2.3
Total fluid volume ml	330	330	330	330	330	330	330	330

*For further details of minerals and vitamins given see Martins et al. (9).

TABLE 2

Fatty Acid Composition of Intralipid® 20%

		Weight %
16:0	Palmitic acid	11.7%
18:0	Stearic acid	3.8%
18:1	Oleic acid	26.5%
18:2ω6	Linoleic acid	49.2%
18:3ω3	Alpha-linolenic acid	7.7%
	Others	1.1%

Average values of ten different batches.

showed values significantly higher in the High fat group at the high energy level than in the other groups.

The serum free fatty acids (Table 3) showed the lowest values in the No fat and Low fat groups. The highest FFA values were found in both High fat groups.

The serum total cholesterol and phospholipid levels also paralleled the amount of fat administered (Table 3). The levels were elevated significantly in the Medium fat and High fat groups compared with the No fat and Low fat groups. The cholesterol ester levels (Table 3) were significantly lower in the Medium fat and High fat groups regardless of energy supply. This was most pronounced in the 350 High fat group.

Fatty Acid Pattern of Tissue Lipids

Saturated fatty acids. Increasing the amount of fat from 0% to 60% of the non-protein energy infused generally was followed by a decrease in the levels of palmitic acid in the tissues studied.

Monosaturated fatty acids. In our data, 18:1 represents the sum of 18:1ω9 (oleic acid) and 18:1ω11 (vaccenic acid). As for the saturated fatty acids, the higher levels of palmitoleic and oleic acid were detected in the No fat and Low fat groups. The levels of palmitoleic acid showed a steeper fall when the amount of fat was increased in the infusions, when compared with the levels of oleic acid.

Polyunsaturated fatty acids. 18:2ω6. In the adipose tissues, significantly lower values of linoleic acid were observed in the No fat and Low fat groups, when compared with the Medium fat and High fat groups. However, the last 2 groups showed similar values at both energy levels. In the muscle and liver tissues, lower values also were observed in the No fat and Low fat groups, but the levels of linoleic acid were generally higher in the High fat than in the Medium fat groups. The No fat groups showed low levels of linoleic acid with the lowest level observed at the higher energy level.

20:4ω6. In the liver and muscle tissue, arachidonic acid was not only decreased in the groups receiving no fat but also in the group receiving high fat and high energy supply. The levels tended to be lower at the higher energy level.

20:3ω9. The eicosatrienoic acid was detected only in the liver samples. The highest values were seen in the No fat groups at both energy levels. At the high energy level eicosatrienoic acid was more significantly elevated.

TABLE 3

Serum Lipids After Two Days of Continuous Infusions of TPN in Rats (Mean values ± SEM)

Groups	n	TG mmol/l	Total cholesterol mmol/l	Cholesterol ester %	PL mmol/l	FFA[f] μmol/l
270 kcal/kg•day						
No fat	(7)	1.7 ± 0.5	1.3 ± 0.1	77 ± 3	1.5 ± 0.1	155 ± 25
Low fat	(8)	1.2 ± 0.1	1.4 ± 0.1	72 ± 4	1.6 ± 0.04	247 ± 40
Medium fat	(8)	2.0 ± 0.1[b]	2.0 ± 0.1[a,b,c]	55 ± 1[a,b,d]	2.8 ± 0.1[a,b,c,d]	766 ± 48
High fat	(9)	2.0 ± 0.2[b,e]	3.2 ± 0.1[a,b,e]	55 ± 4[a,b,e]	4.1 ± 0.1[a,b,e]	932 ± 29
350 kcal/kg•day						
No fat	(7)	1.8 ± 0.2[e]	1.5 ± 0.1[d,e]	66 ± 2[d,e]	1.8 ± 0.04[a,d,e]	268 ± 68
Low fat	(8)	1.8 ± 0.1[b,e]	1.5 ± 0.1[d,e]	74 ± 2[d,e]	1.6 ± 0.1[d,e]	183 ± 30
Medium fat	(8)	2.0 ± 0.3[e]	2.5 ± 0.1[e]	48 ± 1	3.3 ± 0.1[e]	908 ± 63
High fat	(9)	3.3 ± 0.4	4.4 ± 0.1	40 ± 4	5.1 ± 0.2	1100

[a]Significant difference to 270 No fat.

[b]Significant difference to 270 Low fat.

[c]Significant difference to 270 High fat.

[d]Significant difference to 350 Medium fat.

[e]Significant difference to 350 High fat.

[f]Due to limited amounts of blood, only a few animals in each group were analyzed.

TABLE 4

Total Fatty Acid Composition in the Liver of Rats Receiving Various TPN for 10 Days (Mean values ± SEM)

Fatty acid, number of carbons and double bonds	Oral controls* n = 12	270 kcal/kg•day				350 kcal/kg•day			
		No fat n = 7	Low fat n = 8	Medium fat n = 8	High fat n = 9	No fat n = 7	Low fat n = 8	Medium fat n = 8	High fat n = 9
16:0	19.0	23.1 ± 0.3[a]	25.3 ± 0.5[a]	18.9 ± 0.3[a,b]	17.8 ± 0.3[a]	31.5 ± 0.4[a]	26.8 ± 0.4[a]	18.9 ± 0.3[a,b]	23.0 ± 0.6[a]
18:0	13.7	18.3 ± 0.5[a,b]	19.2 ± 0.6[a,b]	23.1 ± 0.3[a]	25.8 ± 1.0[a]	16.0 ± 1.0[a]	18.1 ± 0.3[a,b]	18.4 ± 0.9[a,b]	18.8 ± 1.3[a,b]
16:1	1	5.3 ± 0.3[a]	2.3 ± 0.2[a]	n.d.[a,b]	n.d.[a,b]	7.0 ± 0.4[a]	3.4 ± 0.1[a]	0.8 ± 0.2[a]	n.d.[a,b]
18:1	13.3	17.9 ± 0.5[a]	12.7 ± 0.5[a,b]	12.5 ± 0.4[a,b]	11.0 ± 0.4[a,b]	23.4 ± 1.4[a]	14.6 ± 0.4[a,c]	15.2 ± 1.0[a,c]	15.0 ± 0.8[a,c]
18:2ω6	23.8	9.0 ± 0.7[a]	17.4 ± 0.2[a]	24.2 ± 0.2[a]	26.5 ± 0.4[a,b]	4.5 ± 0.8[a]	15.1 ± 0.3[a]	26.8 ± 0.8[a,b]	30.2 ± 0.7[a]
18:3ω3	1.5	n.d.[a]	n.d.[a]	0.8 ± 0.1[a,b]	0.7 ± 0.1[a,b]	n.d.[a]	n.d.[a]	1.2 ± 0.1[a,b]	1.3 ± 0.1[a,b]
20:3ω9	–	2.0 ± 0.3[a]	0.6 ± 0.2[a,b]	0.6 ± 0.2[a,b]	0.3 ± 0.1[a,b]	3.7 ± 0.3[a]	0.4 ± 0.1[a,b]	0.4 ± 0.1[a,b]	0.4 ± 0.1[a,b]
20:4ω6	15.9	15.3 ± 0.8[a,c]	19.0 ± 0.5[a,b]	18.0 ± 0.6[a,b]	16.6 ± 0.6[a,c]	10.0 ± 1.0[a,d]	17.8 ± 0.4[a]	14.9 ± 0.7[a,c]	10.2 ± 0.8[a,d]

n.d.–not detected or below 0.3%.

*Meurling et al. (24).

[a]Values sharing a common superscript "a" show a statistical significant difference. If "a" is followed by b, c or d see footnote. The comparisons were made with all groups within the same energy level and the corresponding group at the other energy level.

[b]Values are not different in groups sharing this letter.

[c]Values are not different in groups sharing this letter.

[d]Values are not different in groups sharing this letter.

TABLE 5

Total Fatty Acid Composition of Tibialis Anterior Muscle in Rats Receiving Various TPN for 10 Days (Mean values ± SEM)

Fatty acid, number of carbons and double bonds	270 kcal/kg•day				350 kcal/kg•day			
	No fat n = 7	Low fat n = 8	Medium fat n = 8	High fat n = 9	No fat n = 7	Low fat n = 8	Medium fat n = 8	High fat n = 9
16:0	25.3 ± 1.7	25.9 ± 0.8	23.0 ± 0.7	22.2 ± 0.9	25.5 ± 0.5	25.8 ± 0.7	24.8 ± 0.3	21.7 ± 0.3
18:0	14.4 ± 0.9	16.0 ± 0.6	13.6 ± 0.7	15.1 ± 0.5	11.7 ± 0.4	13.8 ± 0.8	13.7 ± 1.1	14.0 ± 0.7
16:1	3.3 ± 0.4[a]	2.0 ± 0.1[a,b]	1.7 ± 0.2[a,b]	1.4 ± 0.3[a,b]	6.2 ± 0.3[a]	3.4 ± 0.3[a,b]	3.4 ± 0.5[a,b]	1.9 ± 0.2[a]
18:1	17.3 ± 1.2[a,b]	13.6 ± 0.5[a,c]	15.1 ± 0.9[a,b]	12.7 ± 0.9[a,c]	21.2 ± 0.6[a]	15.4 ± 0.8[a,b]	17.3 ± 1.0[a,b]	15.6 ± 0.8[a,b]
18:2ω6	19.7 ± 0.2[a]	21.6 ± 0.4[a,c]	24.4 ± 0.7[a,b]	23.8 ± 1.0[a,b]	15.7 ± 0.7[a]	21.8 ± 0.5[a,c]	27.8 ± 0.3[a]	31.2 ± 0.4[a]
18:3ω3	n.d.[a]	n.d.[a]	1.2 ± 0.1[a]	0.7 ± 0.1[a]	0.7 ± 0.1	0.9 ± 0.1	1.5 ± 0.2	1.1 ± 0.1
20:4ω6	11.5 ± 0.8[a,b]	12.7 ± 0.4[a,b]	10.0 ± 0.9[a,c]	13.8 ± 1.1[a,b]	7.9 ± 0.3[a,d]	10.4 ± 0.7[a,c]	8.5 ± 0.8[a,d]	6.9 ± 0.4[a,d]

n.d.—not detected or below 0.3%.

[a]Values sharing a common superscript "a" show statistical difference. If "a" is followed by b, c or d see footnote. The comparisons were made with all groups within the same energy level and the corresponding group at the other energy level.

[b]Values are not different in groups sharing this letter.

[c]Values are not different in groups sharing this letter.

[d]Values are not different in groups sharing this letter.

TABLE 6

Total Fatty Acid Composition in Epididymal Fat Tissue of Rats Receiving Various TPN for 10 Days (Mean values ± SE)

Fatty acid, number of carbons and double bonds	270 kcal/kg•day				350 kcal/kg•day			
	No fat n = 7	Low fat n = 8	Medium fat n = 8	High fat n = 9	No fat n = 7	Low fat n = 8	Medium fat n = 8	High fat n = 9
16:0	27.5 ± 0.5[a,b]	26.4 ± 0.4[a,b]	23.8 ± 0.4[a,c]	24.5 ± 0.4[a,c]	34.2 ± 0.3[a]	30.8 ± 0.4[a]	26.2 ± 0.4[a,b]	25.3 ± 0.3[a,b]
18:0	3.3 ± 0.1	3.3 ± 0.1	3.6 ± 0.1	3.6 ± 0.1	3.5 ± 0.1	3.2 ± 0.1	3.1 ± 0.02	3.2 ± 0.1
16:1	7.3 ± 0.4[a,b]	6.3 ± 0.4[a,b]	4.9 ± 0.3[a,c]	5.0 ± 0.3[a,c]	12.4 ± 0.1[a]	9.6 ± 0.2[a]	6.7 ± 0.2[a,b]	5.8 ± 0.2[a,b]
18:1	32.2 ± 0.4[a,b]	32.1 ± 0.3[a,b]	29.1 ± 0.1[a,c]	30.3 ± 0.4[a,c]	34.7 ± 0.6[a]	30.1 ± 0.2[a]	29.0 ± 0.2[a,c]	28.6 ± 0.2[a,c]
18:2ω6	22.8 ± 0.6[a]	25.0 ± 0.6[a]	30.5 ± 0.4[a,b]	29.5 ± 0.7[a,b]	10.4 ± 0.5[a]	20.3 ± 0.6[a]	29.0 ± 0.4[a,b]	30.5 ± 0.3[a,b]
18:3ω3	2.2 ± 0.1[a,b]	2.5 ± 0.1[a,b]	3.4 ± 0.1[a]	1.1 ± 0.1[a]	1.1 ± 0.1[a]	2.4 ± 0.1[a,b]	3.7 ± 0.04[a]	4.2 ± 0.1[a]
20:4ω6	0.3 ± 0.01	0.3 ± 0.01	0.4 ± 0.01	0.3 ± 0.02	0.2 ± 0.05	0.3 ± 0.01	0.3 ± 0.02	0.3 ± 0.01
% E.F.A.	25.4 ± 0.6[a]	27.6 ± 0.6[a]	34.3 ± 0.5[a,b,d]	32.6 ± 0.07[a,b]	11.7 ± 0.6[a]	23.0 ± 0.7[a]	33.0 ± 0.4[a,b]	35.0 ± 0.3[a,b,d]

[a]Values sharing a common superscript "a" show statistical difference. If "a" is followed by b, c or d see footnote. The comparisons were made with all groups within the same energy level and the corresponding group at the other energy level.

[b]Values are not different in groups sharing this letter.

[c]Values are not different in groups sharing this letter.

[d]Values are not different from 350 Medium fat.

TABLE 7

Total Fatty Acid Composition in Subcutaneous Fat Tissue of Rats Receiving Various TPN for 10 Days (Mean values ± SE)

Fatty acid, number of carbons and double bonds	270 kcal/kg•day				350 kcal/kg•day			
	No fat n = 7	Low fat n = 8	Medium fat n = 8	High fat n = 9	No fat n = 7	Low fat n = 8	Medium fat n = 8	High fat n = 9
16:0	29.0 ± 0.7[a,b]	26.5 ± 0.5[a,c]	24.8 ± 0.3[a]	23.7 ± 0.3[a]	33.5 ± 0.3[a]	30.5 ± 0.4[a,b]	27.6 ± 0.7[a,c]	25.8 ± 0.3[a,c]
18:0	3.9 ± 0.1	4.1 ± 0.2	3.8 ± 0.1	4.5 ± 0.1	3.7 ± 0.1	3.8 ± 0.1	3.1 ± 0.1	3.5 ± 0.1
16:1	7.9 ± 0.6	6.0 ± 0.5	5.0 ± 0.3	3.8 ± 0.1	11.9 ± 0.3	9.1 ± 0.5	7.7 ± 0.4	6.0 ± 0.3
18:1	32.7 ± 0.7[a,b]	32.6 ± 0.4[a,b]	29.5 ± 0.2[a,c]	32.5 ± 0.5[a,b]	33.8 ± 0.3[a,b]	31.6 ± 0.5[a,b]	28.2 ± 0.1[a,c]	28.3 ± 0.2[a,c]
18:2ω6	18.9 ± 1.0[a]	23.7 ± 0.4[a]	28.6 ± 0.5[a,b]	28.6 ± 0.5[a,b]	12.3 ± 0.4[a]	18.6 ± 1.0[a]	25.9 ± 0.7[a]	28.7 ± 0.5[a,b,c]
18:3ω3	1.5 ± 0.1[a]	2.0 ± 0.1[a,b]	2.8 ± 0.1[a,c]	2.3 ± 0.1[a,b]	1.1 ± 0.1[a]	1.9 ± 0.1[a,b]	3.1 ± 0.1[a,c]	3.7 ± 0.1[a]
20:4ω6	0.3 ± 0.01	0.4 ± 0.01	0.4 ± 0.02	0.4 ± 0.03	0.2 ± 0.02	0.3 ± 0.01	0.4 ± 0.02	0.4 ± 0.02
% E.F.A.	20.7 ± 1.1[a,c]	26.1 ± 0.5[a]	31.8 ± 0.7[a,b]	31.3 ± 0.6[a,b]	13.6 ± 0.4[a]	20.8 ± 1.1[a,c]	29.4 ± 0.8[a]	32.8 ± 0.6[a,b]

[a]Values sharing a common superscript "a" show statistical difference. If "a" is followed by b, c or d see footnote. The comparisons were made with all groups within the same energy level and the corresponding group at the other energy level.

[b]Values are not different in groups sharing this letter.

[c]Values are not different in groups sharing this letter.

[d]Values are not different from 350 Medium fat.

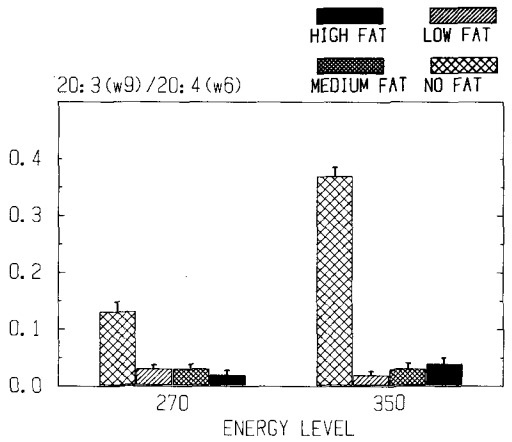

FIG. 1. Triene/tetraene ratios in liver of rats given various TPN (Mean value ± SEM).

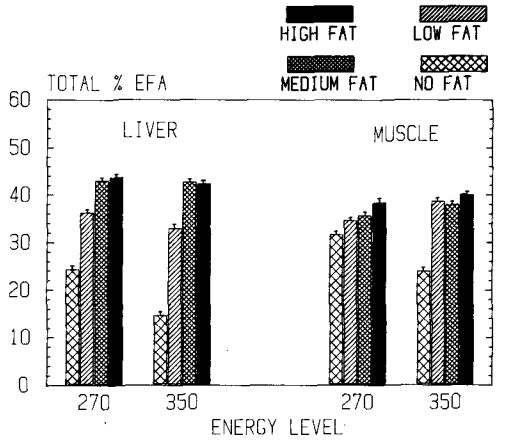

FIG. 2. Total EFA in liver and muscle of rats given various TPN (Mean value ± SEM).

The triene/tetraene ratio (Fig. 1) was significantly elevated in the liver of both groups receiving no supply of fat. A higher energy supply further changed this. In the muscle total EFA was lowered only in the No fat groups.

DISCUSSION

One of the main concerns, when using TPN in clinical practice, is the determination of the optimal dosage of fat in order to avoid a deficiency syndrome or fat overload. It is desirable to reach EFA concentrations as close as possible to those found in the tissues of healthy individuals. This study reports the modulating effect of energy and energy substrate ratios on serum lipid profiles and total fatty acid composition in different tissues of rats submitted to various regimens of TPN for 10 days.

The appearance of an EFA deficiency syndrome is a slow metabolic process that can take up to months to fully develop (15), depending on the type of diet administered, the metabolic state of the animal, its age and growth rate (16). White (17) showed that a biochemical imbalance of the lipid composition in the various organs usually precedes the appearance of clinical symptoms of EFA deficiency.

Given to animals with fast growth, characteristic of this model, the 10-day infusion period was regarded as long enough to show possible differences among groups receiving different treatments. However, with respect to the magnitude of normal fat reserves of a rat, no dramatic changes were expected in the blood lipids or tissue fatty acid pattern of animals deprived of fat for a 10-day period.

The values for serum lipid classes, obtained from samples collected during continuous infusion (day 2), should reflect the steady-state balance between infused substrates and the capacity of the body to clear them from the blood. Since the solutions were infused continuously for 24 hr per day, only small cyclic variations should be expected, and the results can be considered as mean daily values.

In this study, the level of serum TG seemed more influenced by a fat overload than by the absence of fat. When comparing the values obtained with the total amount of TG infused in the different groups (Table 1) it was observed that the clearance capacity of serum TG remained efficient when the amount of TG infused was between 1.5 and 15.3 g TG/kg b.w. and day. The serum clearance capacity became limiting only when 20.4 g TG/kg b.w. was infused per day.

Our study further indicates that the level of energy supplied di not seem to have a major influence on the serum lipids.

Bergström et al. (18) reported high levels of serum FFA in EFA deficient rats. In our experiment the low FFA levels observed during infusion in the No fat and Low fat groups, at both energy levels, could be caused not only by the low fat content in the diet of these groups, but also by the anti-lipolytic effect of circulating high levels of glucose and insulin, isolating the animal from its own fat tissue. This also could be a causative factor for EFA deficiency.

In order to investigate the influence of energy on lipogenesis, the levels of palmitic, palmitoleic, stearic and oleic acids were compared in the 270 kcal Low fat and the 350 kcal Medium fat groups, since these groups were infused with almost the same amount of glucose (Table 2). The results showed no major differences between these groups. In our experi-

ment, lipogenesis seemed to be more dependent on glucose availability than on the energy level or the amount of fat present in the diet.

The patterns of EFA in the adipose tissues studied at the 2 energy levels were similar. The slightly higher concentration of linoleic acid in the Low fat and the Medium fat groups of the peri-epididymal fat possibly indicates a higher metabolic activity in this tissue compared with subcutaneous fat tissue.

According to Alling et al. (19) the muscle is more affected by long periods of fat deprivation than the liver, regarding the EFA composition. In our experiment the liver seemed to be more affected than the muscle, since the concentrations of linoleic and arachidonic acids were lower in the No fat and Low fat groups at both energy levels in the liver tissue.

The 350 kcal No fat rats showed the lowest absolute values of EFA in all the tissues studied. This fact cannot be attributed only to a relative EFA deficiency (higher amounts of saturated and monounsaturated fatty acids synthesized from excess carbohydrate intake). Despite an increased fat content (9) in the livers of No fat rats at the high energy level, the amount of EFA was lowered further compared to the corresponding group at the low energy level.

A lower growth is characteristic of EFA deficiency (1) and, according to Holman (16), a decrease in growth can postpone the appearance of a deficiency syndrome. The experimental animals given a high energy level did grow faster (9), which should give an increased demand of EFA, reflected in a more pronounced biochemical EFA deficiency when the fat administration was limiting. Lower EFA concentration in tissues also was seen in the Low fat groups when the energy was increased. This suggests that the daily requirements of EFA could be expressed in relation to the parameters investigated as an absolute amount of EFA per kg body weight and day or in relation to desired growth, rather than a relative concentration of the daily energy supplied.

The concentrations of arachidonic acid in liver and muscle (Table 5) were significantly lowered with no fat or very high fat (20.4 g/kg) diets. An inhibition in delta-6-desaturase enzyme system could be responsible for this phenomenon caused by different mechanisms. When no fat is given this enzyme is inhibited by low linoleic acid and circulating high levels of glucose. With the high fat diet, it is likely the enzyme activity is inhibited by excess of linoleic acid, resulting in an animal not deficient in linoleic acid but still deficient in arachidonic acid (substrate inhibition). This is supported in the literature by in vitro studies (20).

The 20:3ω9 is detected only rarely in normally fed rats. Rivers et al. (21) consider its presence as a sign of impaired nutritional status. In our experiment we could detect this fatty acid only in the liver. The concentration of eicosatrienoic acid was significantly higher in the two No fat groups, and more pronounced at the higher energy level. This is in agreement with hyperalimentation (22) and hypocaloric nutrition studies (23) and suggests that energy intake is an important factor for EFA status.

The total amount of all EFA in the tissues studied (Fig. 2, Table 6 and 7) showed that maximum incorporation was obtained in the Medium fat and High fat groups regardless of energy intake. No further increased incorporation of total EFA was seen above 7.7 g soybean TG/kg • day. This suggests an optimal linoleic acid intake of 4 g/kg • day.

In conclusion, our study shows that both energy level and substrate composition are important factors for EFA status. When energy is increased the EFA intake can become limiting, resulting in a lowered EFA status.

REFERENCES

1. Burr, G., and Burr, M. (1929) J. Biol. Chem. 82, 345-367.
2. Hansen, A.E.; Wiese, H.F.; Boelsche, A.N.; Haggard, M.E.; Adam, D.J., and Davis, H. (1963) Pediatrics 31, 171-192.
3. Paulsrud, J.R.; Pensler, L.; Whitten, C.F.; Stewart, S., and Holman, R.T. (1972) Am. J. Clin. Nutr. 25, 897-904.
4. Friedman, Z. (1980) Am. J. Dis. Child. 134, 397-408.
5. Collins, F.D.; Sinclair, A.J.; Royle, J.P.; Coats, D.A.; Maynard, A.T., and Leonard, R.F. (1971) Nutr. Met. 13, 150-167.
6. Riella, M.C.; Broviac, J.M.; Wells, M., and Scribner, B.H. (1975) Ann. Int. Med. 83, 786-789.
7. FAO/WHO (1977) FAO Food Nutr. Pap., No. 3.
8. Adam, D.J.; Hansen, A.E., and Wiese, H.F. (1958) J. Nutr. 86, 555-564.
9. Martins, F.M.; Wennberg, A.; Kihlberg, R.; Meurling, S., and Lindmark, L. (1983) Accepted for publication in J.P.E.N.
10. Fletcher, M.J. (1968) Clin. Chim. Acta 22, 393-397.
11. Zlatkiss, A.; Zak, B., and Boyle, A.J. (1953) J. Lab. Clin. Med. 41, 486.
12. Svennerholm, L., and Vonicz, M.T. (1972) Brain Res. 47, 457-468.
13. Richmond, W. (1973) Clin. Chem. 19, 1350-1356.
14. Glass, R.L.; Jenness, R., and Troolin, H. (1965) J. Dairy Sci. 48, 1106-1109.
15. Sinclair, A.J., and Collins, F.D. (1968) Biochim. Biophys. Acta 152, 498-510.
16. Holman, R.T. (1968) in Progress in the Chemistry of Fat and Other Lipids, Vol. 9 (part 2), pp. 275-348.
17. White, H.B.; Turner, M.D.; Turner, A.C., and Miller, R.C. (1973) J. Pediatr. 83(2), 305-313.
18. Bergström, S., and Carlson, L.A. (1965) Acta Physiol. Scand. 64, 479.
19. Alling, C.; Bruce, Å.; Karlsson, I.; Sapia, O., and Svennerholm, L. (1972) J. Nutr. 102, 773-782.

20. Brenner, R. (1981) in Progress in Lipid Research (Holman, R., ed.), Vol. 20, pp. 41-47.
21. Rivers, J.P., and Frankel, T.L. (1981) Br. Med. Bull. 39(1), 59-64.
22. Nubé, M.; Bos, L.P.; v.d. Boomgaard, D.M., and Hekkens, W.T.J.M. (1982) J.P.E.N. 6(2), 134-139.
23. Stegink, L.D.; Freeman, J.B.; Wispe, J., and Connor, W.E. (1977) Am. J. Clin. Nutr. 30, 388-393.
24. Meurling, S., and Roos, K-A. (1981) Acta Chir. Chand. 147, 475-480.

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Effects of Streptozotocin-Induced Diabetes On Phosphoglyceride Metabolism of the Rat Liver

AN-QUOC DANG, FRED H. FAAS and **WILLIAM J. CARTER,** *Veterans Administration Medical Center, 300 East Roosevelt Road, Little Rock, AR 72206, and Department of Medicine, University of Arkansas for Medical Sciences, Little Rock, AR 72205*

ABSTRACT

We have studied the effect of streptozotocin (SZ)-induced diabetes on fatty acyltransferase and phospholipase enzyme activities involved in the synthesis and degradation of rat liver phosphoglycerides. Neither mitochondrial nor microsomal acyl-CoA:glycerol 3-phosphate acyltransferase (GPAT) activity was altered, although insulin treatment stimulated mitochondrial GPAT activity. However, microsomal acyl-CoA:1-acylglycerol 3-phosphate acyltransferase (1-acyl-GPAT) activity increased (24-33 per cent, $p < 0.01$) in the diabetic animals using 3 different acyl-CoA donors: palmitoyl-CoA, oleoyl-CoA and linoleoyl-CoA. SZ-induced diabetes also increased acyl-CoA:1-acylglycerol 3-phosphorylcholine acyltransferase (GPCAT) activity (38-45 per cent, $p < 0.01$) with 3 different acyl-CoA donors: oleoyl-CoA, linoleoyl-CoA and arachidonoyl-CoA. 1-acyl-GPAT and GPCAT activity returned to normal with insulin treatment. In contrast to the increased activity of the microsomal fatty acyltransferases 1-acyl-GPAT and GPCAT, SZ-induced diabetes decreased mitochondrial phospholipase A_2 activity and lysophospholipase activity (49-70 per cent, $p < 0.01$). Insulin treatment of the diabetic rats corrected the decreased lysophospholipase and stimulated phospholipase A_2 activity 35 per cent higher than controls. Since microsomal 1-acyl-GPAT and GPCAT are known to have higher activity toward unsaturated fatty acyl-CoA donors, the increased GPCAT activity coupled with the decreased lysophospholipase activity and the increased 1-acyl-GPAT activity in diabetes would tend to increase the formation of newly synthesized phospholipids containing unsaturated fatty acids. This mechanism plus the decreased fatty acid desaturase (4) may be the factors which alter the fatty acid composition of phosphoglycerides in diabetic rat liver microsomes.
Lipids 19:738-748, 1984.

INTRODUCTION

Recent studies in this (1) and other laboratories (2) have indicated that streptozotocin-induced diabetes changes the fatty acid composition of rat liver microsomal phospholipid. Changes in the phospholipid fatty acid composition due to diabetes also have been observed in a number of tissues other than the liver, such as human platelet (3) and rat heart, kidney, aorta and serum (2). Fatty acids such as linoleic and docosahexaenoic acids were increased and palmitoleic, oleic and arachidonic acids were decreased in the major microsomal phospholipids (1,2) in the diabetic animal. Such fatty acid composition changes have been explained partially by decreases of the Δ_9, Δ_6 and Δ_5 fatty acid desaturases (2,4). Furthermore, it has been found that in the alloxan-induced diabetic rat the conversion of PE to PC by stepwise methylation of PE was decreased (5). Parallel with that decrease was an increase in choline phosphotransferase activity, the enzyme catalyzing the step: CDP-choline + 1,2-diacyl-Sn-glycerol → PC + CMP (5). It was speculated that the increase of the latter step also might bring about changes in fatty acid composition of the microsomal phospholipids in the diabetic rat (5), since the increase of choline phosphotransferase would increase the flux of PC derived from 1,2-diacyl-Sn-glycerol whose secondary acyl groups are predominantly monoene or diene, while the decrease in PE methyltransferase would decrease the conversion of PE to PC, i.e., decrease polyunsaturated fatty acids in PC since PE normally contains substantial amounts of polyunsaturated fatty acids.

Phospholipid fatty acid composition plays an important role in membrane fluidity, enzyme activities and other properties of membranes (6,7). The present study was carried out to determine: a) whether streptozotocin-induced diabetes would change activities of enzymes that are involved in the incorporation (esterification, acylation) of long chain fatty acids into phosphatidic acid and into PC and PE; b) whether diabetes would have an effect on activities of phospholipases that are involved in the degradation of phospholipids, and c) whether these effects can be corrected by insulin treatment. The enzymes studied and reported here are: acyl-CoA: glycerol 3-phosphate acyltransferase (GPAT), acyl-CoA: 1-acylglycerol 3-phosphate acyltransferase (1-acyl-GPAT), acyl-CoA: 1-acylglycerol 3-phosphorylcholine acyltransferase (GPCAT), phospholipase A_2 and lysophospholipase. The first enzyme (GPAT) is known to catalyze a rate-limiting step for the synthesis of phosphatidic acid in

rat liver microsomes (8) and mitochondria (9). After the saturated fatty acid is incorporated into the primary position of Sn-glycerol 3-phosphate by GPAT, 1-acyl-GPAT catalyzes the incorporation of a second unsaturated fatty acid into the secondary position forming phosphatidic acid. GPCAT and phospholipase A_2 form an acylationdeacylation cycle that is known to play an important role in introducing polyunsaturated fatty acids (linolenic and arachidonic acids) into PC and PE. Finally, lysophospholipase catalyzes the degradation of lyso-PC or lyso-PE. Results of this study indeed have pointed out mechanisms by which diabetes can modify the fatty acid composition of phospholipids.

MATERIALS AND METHODS

DL-α-glycerophosphate, L-α-glycerophosphate, fatty acid free bovine serum albumin, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), lysophosphatidylcholine, egg lecithin, 1-palmitoyl-2-oleoyl-Sn-glycerol 3-phosphorylcholine, and fatty acyl-CoAs were obtained from Sigma Chemical Company, St. Louis, Missouri; 1-palmitoylglycerol 3-phosphate and 1-palmitoyl-Sn-glycerol 3-phosphorylcholine were obtained from PL Biochemicals, Milwaukee, Wisconsin. Streptozotocin was donated by Dr. W. Dulin, Upjohn Co., Kalamazoo, Michigan. Precoated silica gel G thin layer chromatography (TLC) plates were from Eastman Kodak Company, Rochester, New York. Precoated silica gel H TLC plates were from E. Merck, Darmstadt, West Germany. [U-^{14}C]-Sn-glycerol 3-phosphate was purchased from ICN Chemical and Radioisotope Division, Irvine, California. 1-palmitoyl-2-[1-^{14}C] oleoyl-Sn-glycerol 3-phosphorylcholine, 1-[1-^{14}C] palmitoyl-Sn-glycerol 3-phosphorylcholine, Aquasol-2 and Omnifluor were from New England Nuclear, Boston, Massachusetts. All other chemicals and reagents were reagent grade and commercially available.

Animals and Their Treatment

Male Sprague-Dawley rats from Charles River Breeding Laboratories were maintained on a Purina Chow diet and water ad libitum. Rats were about 6 weeks old and with a mean body weight of 207 g at the beginning of the experiments. In each experiment they were divided into 3 different groups using 4 or 5 rats per group: controls, diabetics and insulin-treated diabetics. Streptozotocin was dissolved in ice-cold 25 mM citrate buffer, pH = 4.5 and prepared just before use. Diabetes was induced by injection of SZ (75 mg/kg body weight) into the tail vein. Time between SZ injection and sacrifice was 2-3 weeks. Control rats were injected with the citrate buffer vehicle. Ten to 12 units of protamin zinc insulin (PZI) was injected subcutaneously in insulin-treated diabetic rats daily for 3 days before they were killed. Before injections of SZ and insulin and before killing, blood was taken from the tail vein for glucose determination by a commercial glucose oxidase method (Sigma kit 510). Rats were killed by a blow on the head, and the liver was homogenized in ice-cold 0.25 M sucrose (5 ml/g wet weight liver). The homogenate was centrifuged at 1000 × g for 10 min to eliminate cell debris and nuclei. The supernatant was then centrifuged at 6000 × g for 20 min and the resulting pellet washed twice. The mitochondria were resuspended in 0.25 M sucrose and stored at −70 C (10-20 mg protein/ml). The 6000 × g supernatant was centrifuged at 16,000 × g for 20 min and the resulting supernatant was subjected to additional centrifugation at 100,000 × g for 60 min to obtain the microsomal pellet. After the surface of the pellet was washed twice, the microsomal fraction was resuspended in 0.25 M sucrose and stored at −70 C (20 mg protein/ml). Protein was assayed as described by Hartree (10).

Enzyme Assays

Acyl-CoA: glycerol 3-phosphate acyltransferase (GPAT). Both microsomal and mitochondrial GPAT were assayed as described by Yamashita and Numa (11), but in the presence of 2 mM Mg Cl_2 to enhance the enzyme activity as described by Monroy et al. (12). The reaction mixtures (0.35 ml) contained 60 mM Tris HCl, pH 7.4, 0.72 mM DL-α-glycerol 3-phosphate and [U-^{14}C]-Sn-glycerol 3-phosphate (specific activity: 450 dpm/nmol assuming half of the DL-α-glycerol 3-phosphate is Sn-glycerol 3-phosphate), 2 mM $MgCl_2$, and different amounts of acyl-CoA and mitochondrial or microsomal protein as indicated in Figure 1C and 1A, respectively. The reaction mixture was incubated at 37 C for several minutes as indicated in Figure 1B and the reaction terminated by adding 4 ml of chloroform:methanol (2:1, v/v) and 1 ml of 0.2 N hydrochloric acid. After centrifugation, the lower chloroform phase was washed twice with 4 ml of methanol:0.1 N hydrochloric acid (1:1, v/v) each time and counted in 10 ml of Omnifluor cocktail.

Figure 1A shows that when the mitochondrial or microsomal protein content of the reaction mixture was less than 0.5 mg (using either 0.72 mM or 2.0 mM substrate), the activity of both enzymes was proportional to protein concentration. However, the microsomal GPAT showed a decrease in activity when the

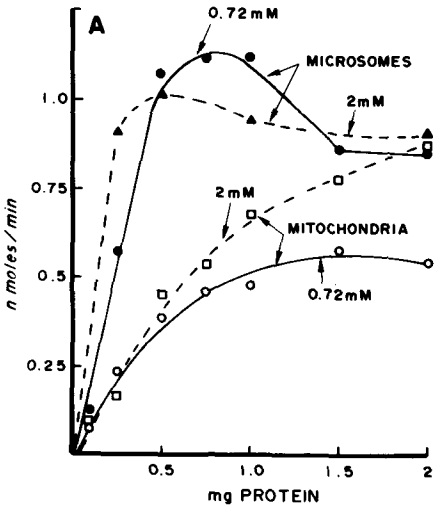

FIG. 1A. Microsomal and mitochondrial GPAT protein-dependent curve. Enzyme activities were assayed as described in Materials and Methods. They were carried out at 2 different substrate concentrations of DL-α-glycerol-3-phosphate (0.72 mM and 2 mM final concentrations), 75 μM acyl-CoA and at different amounts of either mitochondrial or microsomal protein as indicated in figure. Each point was an average of at least 2 determinations.

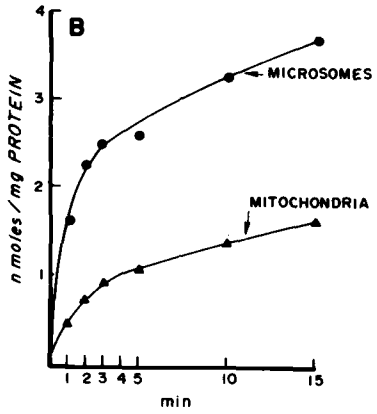

FIG. 1B. Microsomal and mitochondrial GPAT time-dependent curve. Enzyme activities were determined as described in the legend to Figure 1A, and the final concentration of DL-α-glycerol-3-phosphate was 0.72 mM. Each reaction mixture contained 0.5 mg of either microsomal or mitochondrial protein. Time of incubations varied as indicated in the abscissa.

protein content of the reaction mixture was greater than 1 mg. This may be due to the presence of acyl-CoA hydrolase which hydrolyzes the acyl-CoA added as a substrate for the GPAT, since the acyl-CoA hydrolase activity in microsomes is known to be higher than that in

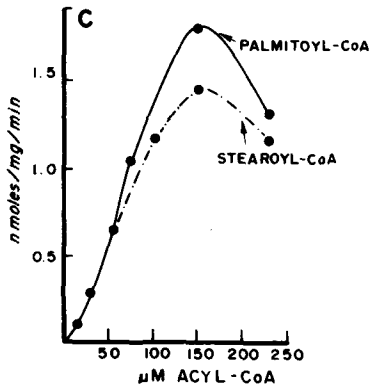

FIG. 1C. Acyl-CoA dependent curve for microsomal GPAT. Microsomal GPAT activity was determined as described in the legend to Figure 1B, but incubation time was 3 min. Final concentration of palmitoyl-CoA or stearoyl-CoA in reaction mixtures was varied as indicated in the abscissa.

mitochondria (13). Both the microsomal and mitochondrial GPAT activity were linear with time of incubation for about the first 3 min (Fig. 1B). When the microsomal GPAT was assayed at different concentrations of palmitoyl-CoA or stearoyl-CoA as indicated in Figure 1C, the highest enzyme activity occurred at 150 μM acyl-CoA. Therefore, in the assays shown in Table 1, reaction mixtures contained 0.5 mg mitochondrial or microsomal protein and 75 μM palmitoyl-CoA and were incubated for 3 min.

Acyl-CoA:1-acyl glycerol 3-phosphorylcholine acyltransferase (GPCAT). This enzyme was assayed either spectrophotometrically by measuring reduction of DTNB at 412 nm by the thiol group of free CoA-SH released or by measuring the conversion of [1-^{14}C]palmitoylglycerol 3-phosphorylcholine to ^{14}C-PC as described by McKean et al. (14). The spectrophotometric assay was carried out at room temperature in a dual beam Aminco, DW-2 UV-vis spectrophotometer set for split beam mode. The reaction mixtures consisted of 100 mM Tris-HCl, pH = 7.4, 0.33 mM DTNB, 25 μM fatty acyl-CoA, 0.1 mM egg lyso-PC and microsomal protein as indicated in Figure 2A in a total volume of 1 ml. Egg lyso-PC was omitted from the reference cuvette. The assay was initiated by the addition of the enzyme to both reference and sample cuvettes and mixed well. The initial reaction rate was recorded continuously at 412 nm for about 1 min and was linear with time. Since both reference and sample cuvettes contained equal amounts of acyl-CoA and microsomes, microsomal acyl-CoA hydrolase activity is cancelled out and the changes in

optical density represent only GPCAT activity. The molar extinction coefficient 13,600 M^{-1} cm^{-1} was used to calculate the enzyme activity. When the radioactive assay was used, the reaction mixture was identical with the spectrophotometric assay described above, but 0.1 mM of 1-[1-^{14}C]-palmitoyl-Sn-glycerol 3-phosphorylcholine (specific activity: 2700 dpm/nmole) was used and the incubation was carried out at 37 C for different periods, as indicated in Figure 2B. The reaction was stopped by adding 4 ml chloroform:methanol (2:1, v/v) and 0.25 ml 2N sulfuric acid to the reaction mixture. After centrifugation, the lower chloroform phase was washed with 4 ml methanol: 0.1 N hydrochloric acid (1:1, v/v). Following the addition of unlabelled egg lecithin and lysophosphatidylcholine carriers, the extracts were spotted on Silica gel H TLC plates which were developed with chloroform:methanol: acetic acid:water (25:15:4:2, v/v) as described by Skipsky et al. (15). The lyso-PC and PC spots were identified by iodine vapor, scraped from the plates and counted in 10 ml Omnifluor cocktail.

In the spectrophotometric assay, the initial rate of enzyme activity was linear with microsomal protein concentration to at least 150 μg protein per assay (Fig. 2A). In the radioactive tracer assay, the enzyme reaction rate was linear with time up to 10 min (Fig. 2B). Thus, the amount of microsomal protein used in both assay procedures was 50 μg, and the incubation time in the radioactive tracer assay was 6 min (Table 1).

Acyl-CoA:1-acylglycerol 3-phosphate acyltransferase (1-acyl-GPAT). This enzyme was assayed spectrophotometrically as described for the GPCAT, except that 0.1 mM 1-palmitoylglycerol 3-phosphate was used as substrate instead of egg lyso-PC. The buffer substrate mixture was sonicated in the presence of fatty acid free BSA (2.0-2.5 mg/ml) and 2 mM $MgCl_2$. The sonification was done with a Bronson sonifier at a setting of 8 (approximately 120 watts) for 1 min or until the substrate was uniformly dispersed.

Figure 3 shows that the initial rate of enzyme activity measured spectrophotometrically was linear with microsomal protein to at least

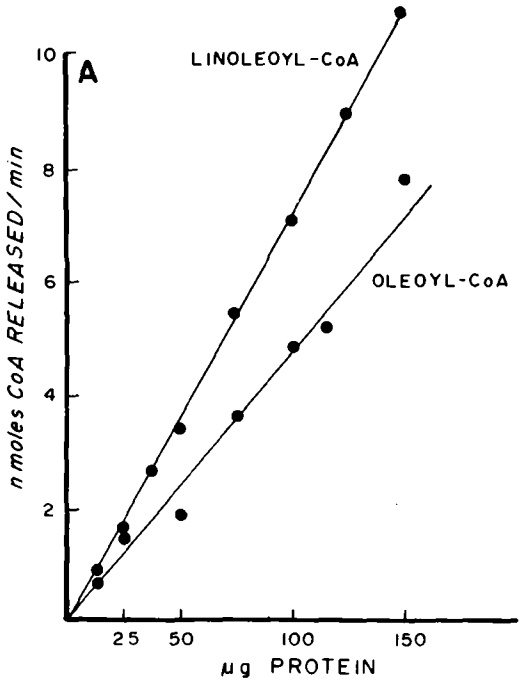

FIG. 2A. Microsomal GPCAT protein-dependent curve. Enzyme activity was determined by spectrophotometric assays as described in Materials and Methods. Linear initial reaction rates were measured at different concentrations of microsomal protein as indicated in the abscissa. The enzyme protein dependent curve was carried out for 2 different acyl-CoA donors: linoleoyl-CoA and oleoyl-CoA. Final concentration of each acyl-CoA in the reaction mixtures was 25 μM. Each point was an average of at least 2 determinations.

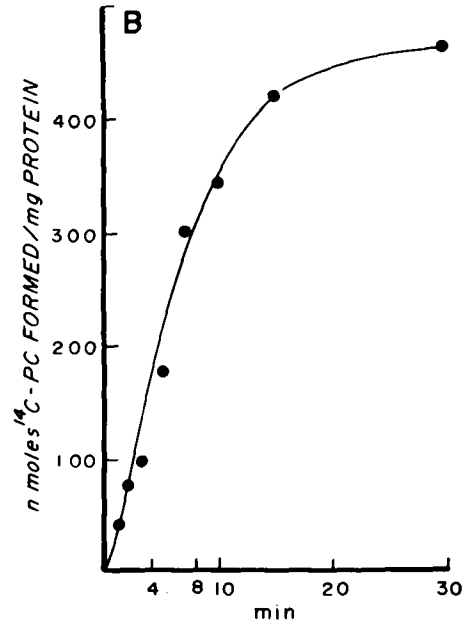

FIG. 2B. Microsomal GPCAT time-dependent curve. Enzyme activity was determined by radioactive assays as described in Materials and Methods. 25 μM oleoyl-CoA was used as acyl-CoA donor. Reactions were started by addition of 50 μg microsomal protein and then stopped at indicated times in the abscissa. Each point was an average of 2 determinations.

TABLE 1

Effects of SZ-Induced Diabetes on Phospholipid Enzyme Activities

Enzymes	Acyl-CoA donors	Enzyme activities (nmoles/mg/min) Controls	Diabetes	% Changed	D + I	% Changed
GPAT (Mc)	Palmitoyl-CoA	2.7 ± 0.2 (6)	2.9 ± 0.2 (6)	+ 7		
GPAT (Mt)	Palmitoyl-CoA	0.45 ± 0.02 (8)	0.40 ± 0.04 (8)	−11		
	Palmitoyl-CoA	0.46 ± 0.03 (8)	0.45 ± 0.03 (8)	− 2	* 0.94 ± 0.13 (9)	+104
1-acyl-GPAT (Mc)	Palmitoyl-CoA	50.2 ± 3.1 (4)	* 62.3 ± 1.5 (4)	+24	48.1 ± 2.2 (5)	
	Oleoyl-CoA	62.2 ± 3.0 (4)	* 80.9 ± 5.2 (4)	+30	60.8 ± 2.6 (5)	
	Linoleoyl-CoA	47.6 ± 3.0 (4)	* 63.2 ± 2.0 (4)	+33		
GPCAT (Mc)	Oleoyl-CoA	49.6 ± 1.7 (6)	* 72.1 ± 3.7 (5)	+45		
	Linoleoyl-CoA	66.0 ± 2.3 (8)	* 91.2 ± 4.1 (9)	+38		
	Arachidonoyl-CoA	97.1 ± 6.0 (6)	+135.6 ± 9.3 (5)	+40		
	Oleoyl-CoA (radioactive tracer assays)	44.1 ± 0.8 (8)	* 54.5 ± 0.8 (8)	+24		
	Arachidonoyl-CoA	64.5 ± 3.8 (4)	* 94.9 ± 6.5 (4)	+48	64.9 ± 2.9 (5)	
[a]Phospholipase A_2 (Mt)		0.53 ± 0.10 (8)	0.36 ± 0.07 (8)	−32	***0.71 ± 0.12 (8)	+ 35
[b]Lysophospholipase		4.17 ± 0.70 (5)	* 1.25 ± 0.20 (5)	−70		
		3.16 ± 0.45 (4)	** 1.61 ± 0.33 (4)	−49	3.44 ± 0.63 (5)	

Enzyme activities were measured in microsomal (Mc) or mitochondrial (Mt) preparations isolated from the livers of diabetic rats, age-matched control rats, and insulin-treated diabetic rats (D + I). Each enzyme activity was determined in triplicate, and numbers of rats studied are shown in ( ). Enzyme activities are shown as means ±SEM. Significant differences between diabetic or insulin-treated diabetic rats versus controls are shown by: *$p < 0.01$, **$p < 0.05$. Significant differences between insulin treated diabetic and diabetic rats are shown by ***$p < 0.05$.

[a]Enzyme activity (nmoles/mg/hr).

[b]Enzyme was assayed on 16,000 × g supernatant.

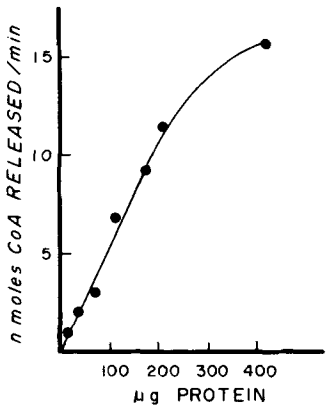

FIG. 3. Microsomal 1-acyl-GPAT protein-dependent curve. Enzyme activity was measured spectrophotometrically as described in Materials and Methods. 25 µM oleoyl-CoA was used as an acyl-CoA donor. Linear initial reaction rates were measured at different concentrations of microsomal protein as indicated in the abscissa. Each point was an average of 2 or 3 determinations.

200 µg per assay. Results in Table 1 were performed at a microsomal protein concentration of 50 µg per assay.

Lysophospholipase. This enzyme was assayed by determining the release of labelled palmitic acid from lysophospholipid. The incubation mixtures (0.5 ml) contained 100 mM Tris-HCl, pH = 7.4, different amounts of 1-[1-^{14}C]palmitoyl-Sn-glycerol 3-phosphorylcholine (S.A.: 450-650 dpm/nmole) and 16,000 g supernatant protein as indicated in Figures 4C and 4A, respectively. The reaction was started by adding the enzyme and the reaction mixture incubated at 37 C for the periods indicated in Figure 4B. The reaction was stopped by the addition of 4 ml chloroform:methanol (2:1, v/v) and 0.25 ml 2N sulfuric acid. After extracting ^{14}C-palmitic acid released from the reaction into the chloroform by vortex mixing, the lower chloroform phase was washed twice with 4 ml methanol: 0.1N hydrochloric acid (1:1, v/v). The ^{14}C-palmitic acid was separated from unreacted ^{14}C-lyso-PC substrate by TLC on Silica gel G, developed with the solvent system: n-hexane:diethylether:acetic acid (80:20:1, v/v). In this procedure, the recovery of ^{14}C-palmitic acid was 85-95 per cent. TLC also was carried out on Silica gel H, developed with the solvent system chloroform: methanol:acetic acid:water (25:15:4:2, v/v) as described above for the enzyme GPCAT. In the latter procedure, ^{14}C-PC also was determined, since it was separated from ^{14}C-palmitic acid and ^{14}C-lyso-PC. The amount of ^{14}C-palmitic acid released was similar in both TLC systems.

It appears that lysophospholipase is inhibited either at high protein or high substrate concentration. For assays using 0.16 mM lysophosphatidylcholine (Fig. 4A), enzyme activity was inhibited when the protein content of the reaction mixture was greater than 1 mg. Using 0.8 mg protein per assay, enzyme activity was linear with time of incubation up to 10 min (Fig. 4B). Figure 4C shows that when 0.8 mg protein per assay was used, the enzyme activity was not inhibited at substrate concentrations up to 0.25 mM, but when 0.4 mg protein per assay was used the enzyme activity was inhibited at substrate concentrations greater than 0.12 mM. Thus, we selected 0.16 mM lysophosphatidylcholine and 0.8 mg protein for this enzyme assay with an incubation time of 10 min per assay (Table 1).

Phospholipase A_2. This enzyme was assayed as described by Shakir (16): To 50 µl aliquots of 5 mM 1-palmitoyl-2-[1-^{14}C]oleoyl-Sn-glycerol 3-phosphorylcholine (specific activity = 230-450 dpm/nmole) dissolved in chloroform: methanol (2:1, v/v) were added 70 µl of 0.5 per cent Triton (v/v). The solvent was then evaporated and 130 µl of buffer containing 100 mM glycine-NaOH, pH 9.5, 0.5 M KCl, 5 mM $CaCl_2$ and 2 mM sodium deoxycholate added. The reaction was started by adding 300 µl of rat liver mitochondria in 0.25 M sucrose containing different amounts of protein as indicated in Figure 5A. The reaction mixtures were incubated at 37 C for several hr as indicated in Figure 5B and the reactions terminated by adding 2 ml heptane:isopropanol: 2 N sulfuric acid (5:20:1, v/v). The ^{14}C-oleic acid released into the reaction mixture and the unreacted substrate were extracted into the heptane layer and separated by using 150 mg silicic acid (16). To verify the amount of ^{14}C-oleic acid released, the heptane layer was evaporated and then applied to silica gel G TLC plates and developed with the solvent system: n-hexane:diethylether:acetic acid (80:20:1, v/v) as described above for lysophospholipase. Both of these procedures yielded similar amounts of released ^{14}C-oleic acid.

Figure 5A and 5B indicate that the reaction rate was linear with time up to 3 hr and with mitochondrial protein concentration to 8 mg protein per assay. Therefore, phospholipase A_2 was assayed with incubation time of 3 hr in the presence of 4 mg mitochondrial protein per assay (Table 1).

Statistical Analysis

The results of replicate experiments were pooled for statistical analysis, and the number of animals used for each assay are indicated in

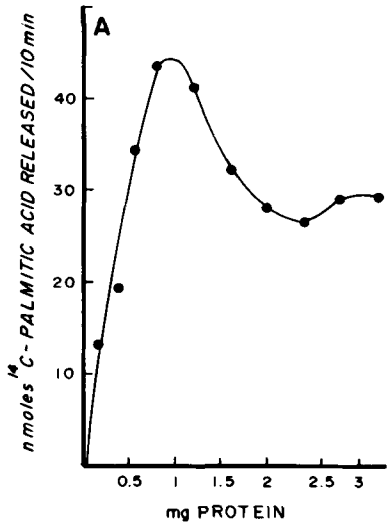

FIG. 4A. Lysophospholipase protein-dependent curve. Enzyme activity was measured as described in Materials and Methods. Reaction mixtures contained 0.16 mM of substrate 1-[1-^{14}C] palmitoyl-Sn-glycerol 3-phosphorylcholine and different amounts of 16,000 X g supernatant protein as indicated in the abscissa. Incubations were done at 37 C for 10 min. Each point was an average of 2 determinations.

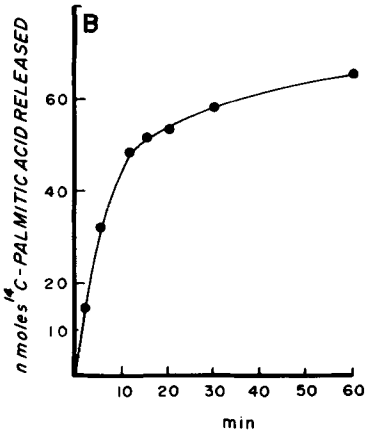

FIG. 4B. Lysophospholipase time-dependent curve. Enzyme activity was measured as described in the legend to Figure 4A except that 0.8 mg of protein was used and incubation times were varied as indicated in the abscissa.

the tables. Significant differences between groups were determined by using Student's t test.

RESULTS

The diabetic rats had a significant increase in blood glucose (diabetic: 477 mg/dl ± 8, control: 130 ± 6 mg/dl) and a significant decrease (25 per cent, $p < 0.001$) in weight gain 2-3 weeks after induction of diabetes as compared to corresponding control rats. The untreated diabetic rats also showed overt polyuria, polyphagia and polydypsia. Daily treatment of the diabetic rats with 10-12 units of protamine zinc insulin for 3 days corrected these symptoms and decreased their blood glucose to control levels.

Enzyme Characteristics

The microsomal GPAT enzyme activity was 3 to 6 times higher than that of the mitochondrial GPAT (Table 1), which had an activity similar to that reported by Grosjean and Haldar (17). The activity of the microsomal GPAT has been separated from the microsomal 1-acyl-GPAT (11). As seen in Figure 3 and Table 1, the 1-acyl-GPAT enzyme activity is about 20 times higher than microsomal GPAT. This is similar to the report of Lands et al. (18). Table 1 also shows that the 1-acyl-GPAT had a

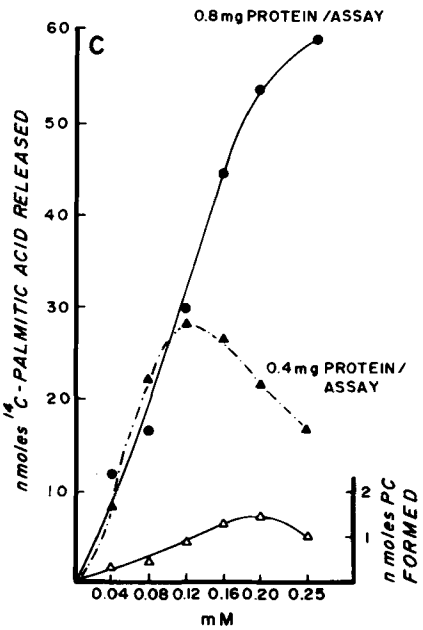

FIG. 4C. Lysophospholipase substrate-dependent curve. Enzyme activity was determined as described in the legend to Figure 4A and 4B, but 2 different amounts of protein were used: either 0.8 mg (●—●—●) or 0.4 mg (▲-•-•-▲-•-•-▲) as indicated in the figure, and the final substrate concentrations were varied as indicated in the abscissa. Incubation time was 10 min. In the assays with 0.4 mg protein/assay, the formation of ^{14}C-phosphatidylcholine also was determined and plotted on the bottom line (△—△—△). This line represents lysophosphatidylcholine:lysophosphatidylcholine transacylase activity. Note that a smaller scale was used for the transacylase activity as indicated in the right ordinate.

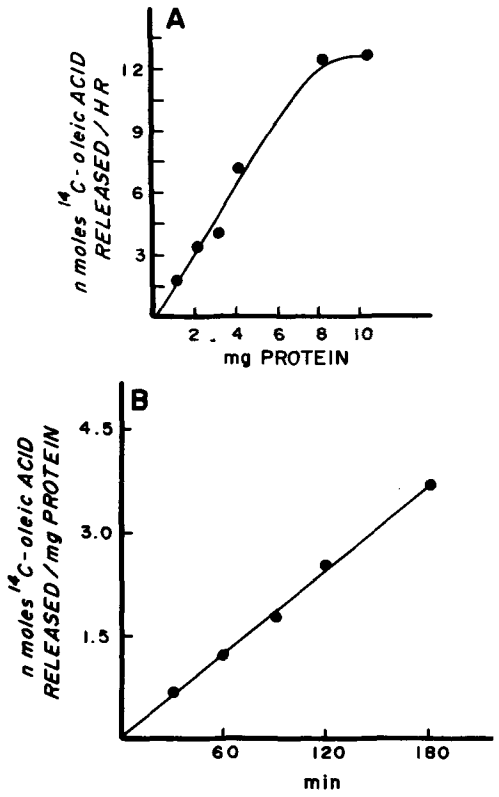

FIG. 5A. Phospholipase A_2 protein-dependent curve. Enzyme activity was determined by the Shakir procedure (16) as described in Materials and Methods. Incubations were carried out for 3 hr at 37 C in the presence of different amounts of mitochondrial protein as indicated in the abscissa. Each point was an average of 2 determinations.

FIG. 5B. Phospholipase A_2 time-dependent curve. Enzyme activity was determined as described in the legend to Figure 5A, but 4 mg mitochondrial protein were used and incubation times were varied as described in the abscissa.

higher activity toward the monounsaturated fatty acyl-CoA (oleoyl-CoA) than toward the saturated acyl-CoA (palmitoyl-CoA). Similar observations were made by Lands et al. (18) and by Yamashita et al. (19) using the partially purified enzyme.

The microsomal enzyme GPCAT has been separated from microsomal 1-acyl-GPAT (19). The activity of the GPCAT enzyme toward polyunsaturated fatty acyl-CoA donors occurred in the following order: arachidonoyl-CoA > linoleoyl-CoA > oleoyl-CoA (Table 1). Similar results were obtained by Lands et al. (18). The radioactive tracer technique gave results similar to the spectrophotometric assays (Fig. 2B and Table 1), supporting the validity of the spectrophotometric assays.

Subcellular fractionation of rat liver homogenates has shown that 82 per cent of lysophospholipase activity is found in the microsomal and protein soluble fractions (20). In this study the enzyme was assayed in 16,000 × g supernatants. Substrate inhibition seen in the lysophospholipase assay (Fig. 4C) has previously been reported for this enzyme in rat liver 100,000 g supernatant or in rabbit heart microsomes by van den Bosch et al. (20) and Gross and Sobel (21), respectively. These authors explained that the unusual enzyme properties of lysophospholipase may be due to the different lysophospholipid density existing in the microsomal membrane (21) or to the expression of "protein entities" with different lysophospholipase activities (22).

It has been demonstrated that lysophospholipase in rat lung cytosol has the capability to transfer the fatty acyl moiety from one lysophosphatidylcholine molecule to another with the formation of phosphatidylcholine, i.e., transacylase activity (23). However, in the 16,000 g supernatant from rat liver, the transacylase activity was only 1/30th the optimal activity of lysophospholipase (lower line of Fig. 4C).

Effects of Streptozotocin-induced Diabetes on Enzyme Activities

After the optimal assay conditions were determined for each enzyme (see Methods), the effect of streptozotocin-induced diabetes on enzyme activities was studied. The results obtained in non-diabetic controls, 3-week diabetic rats and in insulin-treated diabetic rats are shown in Table 1.

Table 1 shows that both mitochondrial and microsomal GPAT were not affected by diabetes. Although a previous study reported that specific activity of microsomal GPAT from diabetic animals was increased 58 per cent over controls (24), the present study showed no significant change in GPAT, in agreement with Whiting et al. (25) (Table 1). Bates and Saggerson (24) reported that the diabetic mitochondrial GPAT was decreased 24 per cent over controls. However, the present study indicated only an 11 per cent decrease compared to controls, and this small decrease was not reproducible. Nevertheless, in the insulin-treated diabetic rats, the mitochondrial GPAT activity increased 104 per cent over controls. This suggests that mitochondrial GPAT can be induced by insulin and is consistent with results obtained from a perfused liver system (26).

Although diabetes did not change GPAT activity, it stimulated microsomal 1-acyl-GPAT

activity (24-33 per cent, $p < 0.01$). This increase was observed using 3 different acyl-CoA donors: palmitoyl-CoA, oleoyl-CoA and linoleoyl-CoA. Insulin treatment of diabetic rats for 3 days reduced the microsomal 1-acyl-GPAT to the control level. Microsomal 1-acyl-GPAT is known to catalyze the esterification of the monoene and diene fatty acids into phosphatidic acid (27), whereas the polyunsaturated fatty acids such as eicosatrienoic (C20:3) and arachidonic (C20:4) acids are esterified mainly by the microsomal GPCAT. Therefore, studies of the latter enzyme were carried out. Experimental diabetes also increased GPCAT activity 38-45 per cent above control levels, $p < 0.01$. The increased GPCAT activity also was found with 3 different acyl-CoA donors: oleoyl-CoA, linoleoyl-CoA and arachidonoyl-CoA. The radioactive tracer experiment with oleoyl-CoA as acyl-CoA donor verified the increase in esterification (24 per cent, $p < 0.01$) by GPCAT in the diabetic rat. Experiments using arachidonoyl-CoA as the acyl-CoA donor showed that the increased GPCAT activity in diabetic rats was corrected by daily injections of insulin for 3 days.

In order to determine whether the acylation-deacylation cycle is affected by diabetes, we examined mitochondrial phospholipase A_2 activity. This enzyme had considerably lower activity (0.5-1 nmole/mg/hr) than other enzymes in the phospholipid metabolic scheme as previously reported (28). The results in Table 1 indicate that phospholipase A_2 activity in the diabetic rats was decreased 32 per cent as compared to controls. Insulin treatment not only restored depressed enzyme activity, but caused a stimulation to a level 35 per cent greater than controls and twice that of untreated diabetics ($p < 0.05$). The stimulation of phospholipase A_2 by insulin also has been demonstrated in isolated rat adipocyte plasma membranes (29). A product of phospholipase A_2 is lysophospholipid, which can be hydrolyzed by the lysophospholipase as well as reacylated by GPCAT. Results in Table 1 show that the lysophospholipase activity in diabetes was markedly decreased (49-70 per cent) and that this decrease was restored to normal by insulin treatment. Since the other product in the phospholipase A_2 assay, 1-palmitoyl-lyso PC, was not identified, it is possible that the apparent decrease in phospholipase A_2 activity might have resulted from a phospholipase A_1 activity followed by lysophospholipase activity.

DISCUSSION

This paper presents the effects of streptozotocin-induced diabetes on several enzyme activities involved in phospholipid synthesis and breakdown. The microsomal acyltransferases 1-acyl-GPAT and GPCAT are known to catalyze the incorporation of unsaturated fatty acids into the secondary position of lysophosphoglycerides such as 1-acyl-Sn-glycerol 3-phosphate and 1-acyl-Sn-glycerol 3-phosphorylcholine to form the corresponding phosphatidic acid and phosphatidylcholine. Both of these enzyme activities were found to be increased in diabetic rat liver microsomes. The increase in GPCAT was accompanied by a significantly decreased lysophospholipase activity which catalyzes the hydrolysis of 1-acyl-Sn-glycerol 3-phosphorylcholine. Thus, the decrease of lysophospholipase coupled with the increase in GPCAT would further stimulate the formation of phospholipids containing polyunsaturated fatty acids, since the decrease of lysophospholipase would increase availability of the substrate 1-acyl-Sn-glycerol 3-phosphorylcholine for the acylation reaction catalyzed by GPCAT. Both the increased enzyme activity of microsomal 1-acyl-GPAT and GPCAT and the decreased enzyme activity of lysophospholipase were corrected by insulin treatment of the diabetic rats. Although starvation and diabetes have many similarities, Pugh and Kates (30) reported that starvation or various diets did not affect GPCAT activity in the rat liver.

The enzyme GPAT which catalyzes the esterification of saturated fatty acids into the primary position of Sn-glycerol 3-phosphate was assayed in both mitochondria and microsomes. The microsomal GPAT is distinguishable from the mitochondrial GPAT because the former is sensitive to sulfhydryl reagents while the latter is not (12). The microsomal GPAT also is known to have high activity toward both palmitoyl-CoA and oleoyl-CoA substrates, whereas the mitochondrial GPAT is much more active with palmitoyl-CoA as substrate as compared to oleoyl-CoA (17). Indeed, the major product formed from mitochondrial GPAT is monoacyl-Sn-glycerol 3-phosphate, while the major product formed from the microsomal GPAT and 1-acyl-GPAT is diacyl-Sn-glycerol 3-phosphate (phosphatidic acid). Thus, the synthesis of phosphatidic acid in liver microsomes proceeds through a sequential acylation of Sn-glycerol 3-phosphate and the synthesis is mediated by 2 distinct acyltransferases: GPAT and 1-acyl-GPAT (11). The microsomal GPAT has been partially purified and separated from the microsomal 1-acyl-GPAT and GPCAT (19). Our results indicate that streptozotocin-induced diabetes exerted different effects on microsomal GPAT and 1-acyl-GPAT, with diabetes causing

stimulation of 1-acyl-GPAT while having no effect on the microsomal GPAT. Previous studies have indicated that microsomal GPAT was either increased (24) or unchanged (25) in diabetes and that mitochondrial GPAT was decreased (24). The findings in this report indicate that the increase in microsomal phosphatidic acid synthesis which has been reported in heart microsomes of diabetic rats (31) may be due to the increase in the 1-acyl-GPAT rather than GPAT. Although mitochondrial GPAT was not substantially changed in the diabetic rats, it was induced by insulin. Thus this study suggests that the mitochondrial GPAT is under regulation by insulin as has been demonstrated previously in the perfused liver (24) and may be reduced in more severely insulin deficient diabetic animals.

Enzyme phospholipase A_2 occurs predominantly in the outer membrane of rat liver mitochondria (32). We have found that mitochondrial phospholipase A_2 activity was decreased in the diabetic animals, and that this decrease was overcorrected by insulin treatment. This observation is consistent with studies in adipose tissue plasma membranes (29) in which phospholipase A_2 was found to be stimulated by insulin whether insulin was added at the time of incubation or preincubated with the membranes. Although GPCAT activity was increased in the diabetic rat liver, the decrease in phospholipase A_2 activity suggests that the deacylation-reacylation cycle in phosphoglycerides is not stimulated in diabetes. However, the increased GPCAT activity coupled with the decreased lysophospholipase activity and the increased 1-acyl-GPAT activity in diabetes all would tend to increase the formation of newly synthesized phospholipids containing unsaturated fatty acids. These changes in enzyme activity alone cannot entirely explain the altered fatty acid composition of phospholipids in diabetes, since the GPCAT and 1-acyl-GPAT activities were increased equally using monounsaturated and polyunsaturated fatty acyl-CoA substrates. However, the diminished fatty acid desaturation in diabetes (4) would diminish the pool of monounsaturated fatty acids and arachidonic acid. Thus, the altered enzyme activities which tend to increase the formation of unsaturated fatty acid containing phospholipids, together with the diminished fatty acid desaturase activity, may explain the alterations in fatty acid composition in the diabetic animal.

ACKNOWLEDGMENT

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REFERENCES

1. Faas, F.H., and Carter, W.J. (1983) Lipids 18, 399-342.
2. Holman, R.T.; Johnson, S.B.; Gerrard, J.M.; Mauer, S.M.; Kupcho-Sandberg, S., and Brown, D.M. (1983) Proc. Natl. Amer. Sci. 80, 2375-2379.
3. Kalofoutis, A., and Lekakis, J. (1981) Diabetologica 21, 540-543.
4. Faas, F.H., and Carter, W.J. (1980) Lipids 15, 953-961.
5. Hoffman, D.R.; Haning, J.A., and Cornatzer, W.E. (1981) Proc. Soc. Exp. Biol. Med. 167, 143-146.
6. Nelson, D.H. (1980) Endocrine Reviews, 1, 180-199.
7. Mathur, S.N.; Simon, I.; Lokesh, B.R., and Spector, A.A. (1983) Biochim. Biophys. Acta 751, 401-411.
8. Lloyd-Davies, K.A., and Brindley, D.N. (1975) Biochem. J. 152, 39-49.
9. Sanchez, M.; Nicholls, D.G., and Brindley, D.N. (1973) Biochem. J. 132, 697-706.
10. Hartree, E.F. (1972) Anal. Biochem. 48, 422-427.
11. Yamashita, S., and Numa, S. (1972) Eur. J. Biochem. 31, 565-573.
12. Monroy, G.; Kelker, H.C., and Pullman, M.E. (1973) J. Biol. Chem. 248, 2845-2852.
13. Berge, R.K., and Farstad, M. (1979) Eur. J. Biochem. 95, 89-97.
14. McKean, M.L.; Smith, J.B., and Silver, M.J. (1982) J. Biol. Chem. 257, 11278-11283.
15. Skipski, V.P.; Peterson, R.F., and Barley, M. (1964) Biochem. J. 90, 374-378.
16. Shakir, K.M.M. (1981) Anal. Biochem. 114, 64-70.
17. GrosJean, C.D., and Haldar, D. (1982) FEBS Letters 145, 281-284.
18. Lands, W.E.M.; Inoue, M.; Sugiura, Y., and Okuyama, H. (1982) J. Biol. Chem. 257, 14968-14972.
19. Yamashita, S.; Hosaka, K., and Numa, S. (1973) Eur. J. Biochem. 38, 25-31.
20. van den Bosch, H.; Aarsman, A.J.; Slotboom, A.J., and van Deenen, L.L. M. (1968) Biochim. Biophys. Acta 164, 215-225.
21. Gross, R.W., and Sobel, B.E. (1982) J. Biol. Chem. 257, 6702-6708.
22. van den Bosch, H. (1982) in Phospholipids, vol. 4 (Hawthorne, J.N., and Ansell, G.B., eds.), Elsevier Biochemical Press, The Netherlands, pp. 313-357.
23. Vianen, G.M., and van den Bosch, H. (1978) Arch. Biochem. Biophys. 190, 373-384.
24. Bates, E.J., and Saggerson, D. (1977) FEBS Letters 84, 229-232.
25. Whiting, P.H.; Bowley, M.; Stuston, R.G.; Pritchard, P.H.; Bridley, D.N., and Hawthorne, J.N. (1977) Biochem. J. 168, 147-153.
26. Bates, E.J.; Topping, D.L.; Sooranna, S.P.; Saggerson, D., and Mayes, P.A. (1977) FEBS Letters 84, 225-228.
27. Hill, E.E., and Lands, W.E.M. (1968) Biochim. Biophys. Acta 152, 645-648.
28. Waite, M., and van Deenen, L.L.M. (1967) Biochim. Biophys. Acta 137, 498-517.
29. Bereziat, G.; Wolf, C.; Colard, O., and Polonovski, J. (1978) in Enzymes of Lipid (Gatt, S.; Freysz, L., and Mandel, P., eds.), Plenum Press, New York, pp. 191-199.
30. Pugh, E.L., and Kates, M. (1984). Lipids 19, 48-55.

31. Murthy, V.K., and Shipp, J.C. (1980) J. Mol. and Cell. Cardiol. 12, 299-309.
32. Nachbaur, J.; Colbeau, A., and Vignais, P.M. (1972) Biochim. Biophys. Acta 274, 426-446.

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The Effect of Neurotensin on the Concentration of Cholesterol and Bile Acids in the Guinea Pig

LUDVIK PERIC-GOLIA*, CLARK F. GARDNER, DANA E. WILSON and **MILENA PERIC-GOLIA,** *Veterans Administration Medical Center, Salt Lake City, UT 84148, and Departments of Pathology and Medicine, University of Utah School of Medicine, Salt Lake City, UT 84132*

ABSTRACT

In guinea pigs, total plasma cholesterol concentrations increased above the control values after single intravenous injections and after 3 days of continuous subcutaneous administration of neurotensin (NT). A high dose of NT (125 pmol/100 g body weight) induced tachycardia and severe respiratory distress; the lowest dose (1.25 pmol/100 g body weight) had the greatest hypercholesterolemic effect 15 min after the injections. The bulk of the total plasma cholesterol was in low density lipoprotein fractions. Cholesterol increased in the same fractions after intravenous administrations of NT. NT induced a decrease in the cholesterol content in the ileum but did not affect significantly the cholesterol content in the liver, kidneys or adrenals. In 48-hr fasted controls, plasma cholesterol concentration and cholesterol content in the liver, kidneys, adrenals and terminal ileum increased; after intravenous injections of NT, plasma cholesterol concentration further increased but cholesterol content of the liver, kidneys and ileum decreased. In fed animals, the concentration of the biliary taurochenodeoxycholic acid increased above the control values 5 and 35 min after the intravenous injections of NT. In fasted controls, the total concentration of bile acids was higher than in fed controls, but only the concentration of taurochenodeoxycholic acid further increased after the injections of NT. Proportionately more taurochenodeoxycholic acid than cholesterol was present in bile after the intravenous injections of NT. These data are consistent with the hypothesis that NT has a regulatory role in intestinal cholesterol transport.
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INTRODUCTION

Neurotensin (NT) originally was isolated from the bovine hypothalamus, but later it also was found in considerable concentration in the small intestine (1). Initially, hypotension, increased secretion of ACTH, LH and FSH, and hyperglycemia were described as the major biological effects of NT (1,2). We reported that single intravenous injections of NT induced significant transient hypercholesterolemia in rats, not mediated by the adrenal glands or the pituitary (3).

In our preliminary studies, the hypercholesterolemic response to intravenously injected NT was even more pronounced in guinea pigs than in rats (4). In the present investigation, we examined the duration and dose-dependence of the hypercholesterolemic response to NT, and the effects of continuous administration of NT. Considering the abundance of NT in the wall of the small intestine coexisting with synthesis and assembly of some cholesterol transporting lipoproteins (5), we have suggested that NT may regulate the intestinal transport of cholesterol (3). A study of NT-like immunoreactivity in rats also suggested that NT may be involved in lipid homeostasis (6). To test this hypothesis further, we compared the changes of cholesterol concentrations in the plasma and ileum before and after the intravenous administrations of NT and began to characterize changes in the plasma lipoproteins in the same animals. To evaluate the possible influence of the contents of the intestinal lumen on the hypercholesterolemic effect of NT, we performed these experiments in fed and 48-hr fasted guinea pigs. NT had no effect on the rate of 7α-hydroxylation of cholesterol (7). In the present investigation we examined the effect of NT on the concentrations of cholesterol and bile acids in gall bladder bile.

*To whom correspondence should be addressed.

MATERIALS AND METHODS

Single Injections of NT

NT was purchased from Bachem, Inc., Torrance, California. Its authenticity was confirmed by analysis for amino acid sequence and purity. About 4-month-old male Hartley guinea pigs (280-320 g) from Simonsen Laboratories, Inc., Gilroy, California or from Elm Hill, Chatsworth, Massachusetts, were kept in controlled en ironment animal quarters for at least a week before the experiments. They had free access to Purina guinea pig chow, fresh green vegetables and water until about 2 hr before the experiments. The animals were killed with a neck clamp 5, 15, 25 or 35 min after

injections into the ear veins of 12.5 pmol NT/100 g body weight in 0.05 ml saline (0.9% NaCl)/100 g body weight. In a subsequent experiment testing the effects of different doses of NT, one group of guinea pigs received 125 pmol NT/100 g body weight intravenously and another group received 1.25 pmol NT/100 g body weight, both 15 min before they were killed. Control animals received intravenously 0.05 ml saline/100 g body weight.

Continuous Administration of NT

Because continuous intravenous administration of NT for several days is impractical in guinea pigs, we chose the subcutaneous route by means of osmotic mini-pumps purchased from Alza, Palo Alto, California. The performance of the mini-pumps was tested by measuring the release of the Evans blue dye and by determining the in vitro discharge of NT by radioimmunoassay. Mini-pumps were filled only with saline for the controls or with NT dissolved in saline in a concentration that discharged about 10 pmol NT/100 g body weight/30 min, were implanted subcutaneously on the backs of the animals, and left in place for 3 or 5 days. After these 2 time periods, the animals were killed with a neck clamp.

Fasting

In the fasting study, fed controls had free access to Purina guinea pig chow, fresh green vegetables and water until about 2 hr before the experiments. Fasted animals were without food 48 hr before the experiments but had free access to water. The animals were killed with a neck clamp 15 min after the intravenous injections of 12.5 pmol NT/100 g body weight dissolved in 0.05 ml saline/100 g body weight. The same numbers of fed and fasted guinea pigs were killed in the same manner 15 min after the intravenous injections of 0.05 ml saline/100 g body weight.

Plasma and Tissue Cholesterol

In all experimental groups, blood was obtained by cardiac puncture with heparinized syringes immediately after the animals were killed. Plasma was separated after centrifugation for 30 min at 2,000 x g at room temperature and processed immediately. Bile was obtained by the aspiration of the gall bladders. Liver, kidneys, adrenal glands and the distal portion of the ileum were removed and washed with cold saline. Bile and tissues were immediately frozen at −20 C for further processing shortly thereafter. Cholesterol content in the tissue was determined only in the animals killed 15 min after the injections of 12.5 pmol NT/100 g body weight.

About 200 mg of each tissue was cut with a razor blade into small pieces and then homogenized in a motor-driven high speed homogenizer. Total cholesterol concentrations in the homogenates and aliquots of the plasma samples were determined by the method of Weigensberg and McMillan (8). The washed digitonide was redissolved in methanol and the absorbance of cholesterol measured at 202-203 nm in a Beckman DK-2A recording spectrophotometer. Samples of known concentrations of purified (9) authentic cholesterol (Calbiochem, La Jolla, California) were processed simultaneously by the same method. The intensity of the spectrophotometric absorbance of these samples was used to calculate the plasma and tissue cholesterol concentrations. The recoveries of cholesterol by this method compare very favorably with other methods (10).

Lipoprotein Cholesterol

Due to an insufficient amount of plasma in other groups, separation of lipoproteins was done only in the plasma of fed guinea pigs that received intravenously 12.5 pmol NT/100 g body weight. Plasma samples remaining after the determinations of total cholesterol (8) were pooled. Thus one 7.5 ml control sample (5

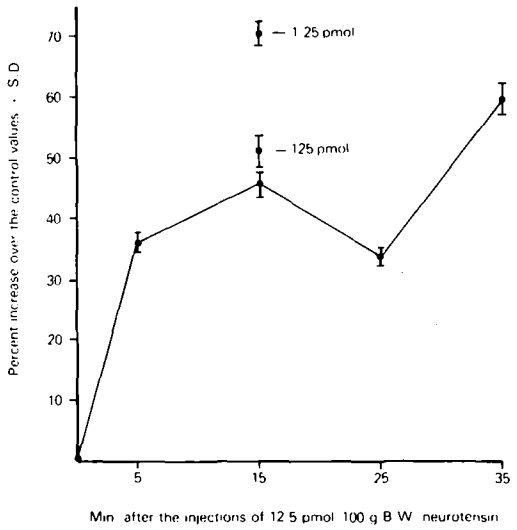

FIG. 1. Per cent increase of the total plasma cholesterol concentrations above the control values in guinea pigs after intravenous injections of 12.5 pmol NT/100 g body weight. The effect of lower and higher doses was examined only 15 min after the injections. Fourteen guinea pigs were used in the control group and at least 8 guinea pigs in each experimental group.

animals) and two 7.5 ml NT samples (3 animals each) were obtained. Each of these samples was diluted to 10 ml by addition of 0.5 ml H_2O and 2.0 ml saline. In these samples, lipoproteins were separated by density gradient ultracentrifugation in a Ti50 vertical rotor (Beckman Instruments Inc., Palo Alto, California). Density gradients were generated during ultracentrifugation at 10 C (11) and verified by refractometry at room temperature. Fractions of 1.8 ml each were collected by displacement with 75% sucrose solution. Cholesterol concentrations were measured in enzymatic assay using cholesterol esterase/cholesterol oxidase (Beckman Instruments, Carlsbad, California).

Determination of Bile Acids and Cholesterol in Bile

Bile acids in the gall bladder bile were separated by thin layer chromatography (TLC) on Whatman KC_{18} F plates with the solvent system ethanol/0.3 M $CaCl_2$/dimethylsulfoxide (25/25/2) (12). Total cholesterol was determined by the method of Weigensberg and McMillan (8).

The significance of the experimental data was determined by means of Student's t-test.

RESULTS

Effect of Single Injections of NT

Total plasma cholesterol concentration was 30±4 mg/100 ml (mean ± S.D.) in the control animals. In all experimental groups it increased significantly above the control value after the intravenous injections of 12.5 pmol NT/100 g body weight (Fig. 1). Fifteen min after the injections of 125 pmol NT/100 g body weight, plasma cholesterol concentrations increased to 46±9 mg/100 ml (mean ± S.D.; $p < 0.001$ vs control). However, these animals had tachycardia and severe respiratory distress. In animals that received only 1.25 pmol NT/100 g body weight, the plasma cholesterol concentrations increased to 52±8 mg/100 ml (mean ± S.D.; $p < 0.001$ vs control) 15 min after the injections. In the majority of animals, NT induced a significant decrease of the total cholesterol concentration in the terminal ileum 15 min after the injections of 12.5 pmol NT/100 g body weight, but did not significantly affect the concentrations of cholesterol in the liver, kidneys or adrenal glands.

Effect of Continuous Administration of NT

On the third day after the implantation of mini-pumps, cholesterol concentrations were significantly higher in the animals bearing mini-pumps with NT than in those bearing mini-pumps with saline. Five days after the implantation, the plasma cholesterol concentrations were about the same in the animals receiving NT as in the controls (Fig. 2).

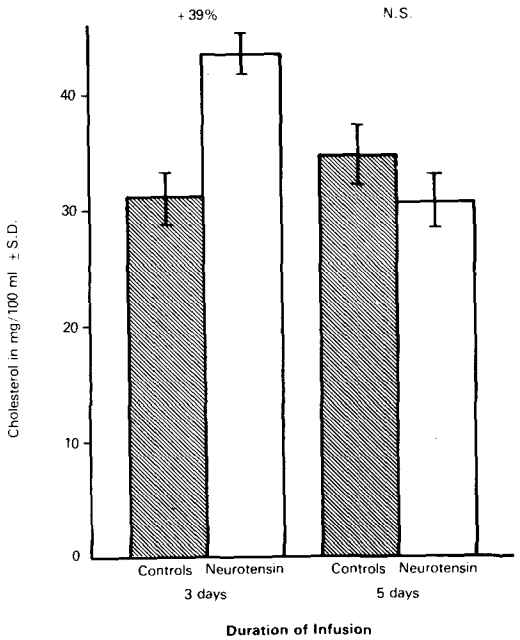

FIG. 2. Total plasma cholesterol concentrations in guinea pigs after continuous administration of NT. Five guinea pigs were used in each control and experimental group. The difference from the corresponding control after 3 days $0.02 < p < 0.01$.

Effect of Fasting

The results of the fasting experiment are presented in Table 1 and 2. Total plasma cholesterol concentrations were significantly higher in fasted animals and further increased after the injections of NT. In 48-hr fasted controls cholesterol concentrations increased in all 4 tissues examined; after the intravenous injections of NT, cholesterol content decreased in the liver, kidneys and ileum. The loss of weight associated with fasting did not change the significance of these data. NT did not affect the adrenal cholesterol concentration.

Lipoproteins

The bulk of total plasma cholesterol was in low density lipoprotein fractions. Cholesterol significantly increased in the same fractions after intravenous administration of NT (Fig. 3).

Biliary Bile Acids and Cholesterol

In fed animals, the concentration of the biliary taurochenodeoxycholic acid signifi-

TABLE 1

Plasma Concentrations of Total Cholesterol in Controls (C) and 15 min After Intravenous Injections of 12.5 pmol NT/100 g Body Weight in Fed Guinea Pigs and After 48-hr Fast

	n	Total Cholesterol in mg/100 ml ± SD	
		C	NT
Fed	10	32 ± 6	45 ± 6[b]
Fasted	16	76 ± 20[a]	104 ± 34[a,c]

[a]P<0.001 vs non-fasted.
[b]P<0.01 vs control.
[c]0.02<p<0.01 vs control.

cantly increased above the control values 5 and 35 min after the intravenous injections of NT (Table 3). In fasted controls, the total concentration of bile acids was higher than in fed controls, but only the concentration of taurochenodeoxycholic acid further increased after intravenous injections of NT (Table 4). No cholic acid was detected in the bile of these guinea pigs. We did not pursue the identification of the minor biliary bile acids. Most gall bladders in the guinea pigs killed 25 min after the injections of NT did not contain a sufficient amount of bile for reliable analysis. In fed guinea pigs, biliary cholesterol was significantly lower 15 min after administration of NT. The ratios between cholesterol and bile acids indicated that NT induced an increased catabolism of cholesterol to bile acids with a preferential conjugation with taurine (Table 4).

DISCUSSION

The increase of plasma cholesterol concentrations in guinea pigs after the intravenous injections of NT in this study (Figure 1) was in accord with the results of our investigations in rats (3,7), which were later confirmed by other investigators (13,14). Hypercholesterolemia induced by 12.5 pmol NT/100 g body weight lasted longer in guinea pigs than in rats. The magnitude of the hypercholesterolemic response was dose-dependent in rats. In guinea pigs, the highest dose of NT (125 pmol/100 g body weight) induced a further increase in the plasma cholesterol concentrations. However, these animals had tachycardia and severe respiratory distress which may have modified the hypercholesterolemic response to NT. Similar adverse effects of high doses of NT have been reported in other species (15). On the other hand, the hypercholesterolemic response to NT was greatest at the lowest dose (1.25 pmol/100 body weight). This observation indicated that NT affects cholesterol transport

TABLE 2

Tissue Concentrations of Total Cholesterol in Controls (C) and 15 min After Intravenous Injections of 12.5 pmol NT/100 g Body Weight in Fed Guinea Pigs and After 48-hr Fast

	n	Cholesterol in mg/100 g ± SD							
		Liver		Kidney		Adrenals		Ileum	
		C	NT	C	NT	C	NT	C	NT
Fed	10	463 ± 39	442 ± 34	618 ± 17	614 ± 63	5,036 ± 1,102	5,276 ± 1,815	390 ± 25	334 ± 54[c]
Fasted	16	572 ± 46[a]	519 ± 28[a,c]	705 ± 63[a]	620 ± 17[c]	6,063 ± 884[a]	5,778 ± 772	607 ± 160[b]	490 ± 116[a,d]

[a]P<0.01 vs non-fasted.
[b]P<0.001 vs non-fasted.
[c]P<0.01 vs control.
[d]0.05<p<0.02 vs control.

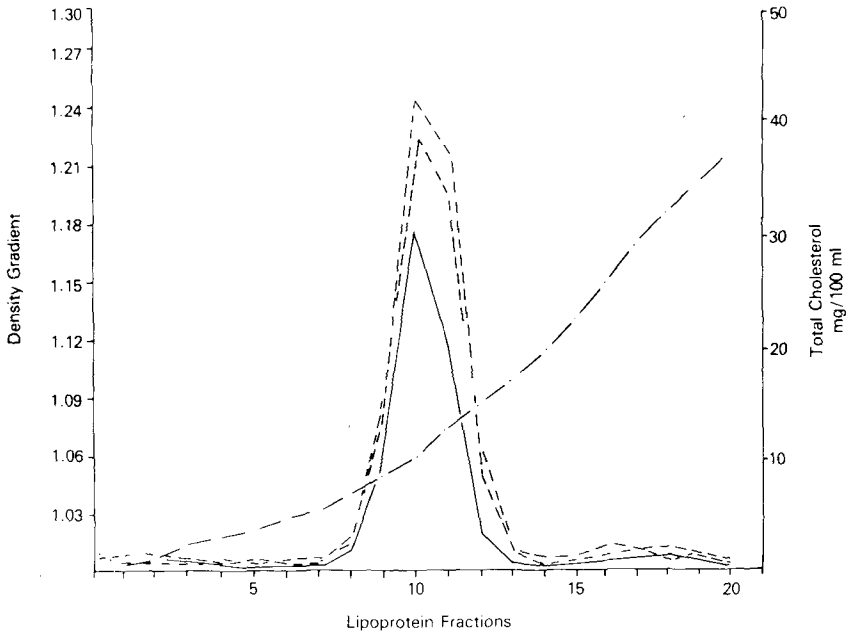

FIG. 3. Fed guinea pigs. Plasma cholesterol in lipoproteins separated by density gradient ultracentrifugation. -·-·-·, density gradient. ——, control. -----, 15 min after intravenous injection of 12.5 pmol NT/100 g body weight.

TABLE 3

Bile Acids in Gall Bladder Bile in Fed Control Guinea Pigs and 5 and 35 min After Intravenous Injections of 12.5 pmol NT/100 g Body Weight

Min after injection	Bile acids in mg/100 ml ± SD			
	Taurochenodeoxycholic		Glycochenodeoxycholic	
	Control	NT	Control	NT
5	126 ± 28[a]	330 ± 124[b]	520 ± 218	607 ± 171
35	126 ± 28[a]	230 ± 62[b]	520 ± 218	550 ± 224

[a]The same controls were used in these 2 successive experiments.
[b]$P<0.01$ vs control.

to the greatest extent when its concentration is only moderately elevated. The increased plasma cholesterol concentration after 3 days of continuous administration of NT indicated a prolonged effect of NT on the plasma cholesterol concentration (Fig. 2). This effect may have been abolished by the fifth day due to an equilibrium between the endogenous and exogenous NT. Delivery of NT by means of minipumps also demonstrated the hypercholesterolemic effect of subcutaneously administered NT.

As in our guinea pigs, fasting has been associated with an increase in plasma cholesterol concentrations in other species (16). The increase of ileal cholesterol in fasted animals is a surprising finding. Some information is available on the lipid concentration in the upper jejunum (17), but no information regarding the ileal concentration of cholesterol in guinea pigs can be found for comparison with our data. It is probable that the rates of lipid absorption and lipoprotein formation differ along the length of the intestinal tract, but only limited comparable data for the distal portion of the intestine are available in any species (18). In fasted animals biosynthesis of cholesterol in the ileum may increase while the synthesis of the cholesterol carrying lipoproteins in the small intestine as well as the amount of lipoproteins carried to the intestine from the liver may decrease. A NT-induced decrease of cholesterol concentration in fed guinea pigs was

TABLE 4

Total Cholesterol and Bile Acids in Gall Bladder Bile in Fed and in Fasted Guinea Pigs 15 min After Intravenous Injections of Saline or 12.5 pmol NT/100 g body Weight

	mg/100 ml ± SD						RATIO			
	Cholesterol (CH)		Taurochenodeoxycholic Ac. (TCD)		Glycochenodeoxycholic Ac. (GCD)		TCD/CH		GCD/CH	
	Saline	NT	Saline	NT	Saline	NT	Saline	NT	Saline	NT
Fed	7.4 ± 1.2	5.8 ± 0.6[a]	165 ± 30	254 ±178	630 ± 140	632 ± 110	22	43	84	108
Fasted	6.2 ± 0.9	7.7 ± 2.3	202 ± 136	603 ± 342[a]	1,049 ± 292[b]	1,000 ± 567[c]	32	78	162	129

[a] $0.05 < p < 0.02$ vs saline.
[b] $P < 0.01$ vs non-fasted.
[c] $0.05 < p < 0.02$ vs non-fasted.

observed only in the ileum. Thus, following the intravenous injections of NT, the decrease of cholesterol concentrations in the ileum both in fed and in fasted animals coincided with the increased plasma cholesterol concentrations. This observation suggests that some of the additional circulating cholesterol may be brought into the plasma pool from the intestine. Such transfer of a portion of the intestinal cholesterol into the plasma would indicate that NT stimulates the rapid transport of cholesterol carrying lipoproteins from the intestinal wall into the blood (19), possibly following the increased absorption of nutrients from the intestinal lumen (20).

In our animals, distribution of cholesterol in low density lipoproteins corresponded to that reported by others (21), and the additional cholesterol in the plasma of guinea pigs treated with NT was distributed in the same lipoprotein fractions.

Chenodeoxycholic acid conjugated with taurine or glycine was the only major biliary bile acid present. The lack of cholic acid was not surprising, since this acid usually is not found in the bile of young guinea pigs (22). The increased biliary concentrations of chenodeoxycholic acid in fasted animals and in 2 groups of fed animals following intravenous injections of NT may be due to the commonly observed variation in the concentrations of bile acids in gall bladder bile (23); more likely, the increase may be due to an enhanced catabolism of cholesterol. Since in rats we did not observe any effect of NT on the 7α-hydroxylation of cholesterol (7), the enhanced catabolic rate probably occurs at a different step of cholesterol metabolism. Conjugation with glycine prevailed in the control bile, but chenodeoxycholic acid that presumably was formed after the administration of NT was conjugated with taurine. This may be due to the greater affinity of taurine conjugates for the hepatic transport system (24) and thus support the suggestion that some chenodeoxycholic acid was newly formed after intravenous administration of NT.

The results of this study further support the putative role of NT in the regulation of cholesterol transport. The mechanism of NT-induced hypercholesterolemia is not known. However, these data suggest that a small increase in the plasma concentration of NT brings about an accelerated transport of cholesterol in the low density lipoprotein fractions from the small intestine into the blood with a probable subsequent increase in the rate of catabolism of cholesterol.

ACKNOWLEDGMENT

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REFERENCES

1. Leeman, S.E. (1980) J. Exp. Biol. 89, 193-200.
2. Carraway, R.E.; Demers, L.M., and Leeman, S.E. (1976) Endocrinology 99, 1452-1462.
3. Peric-Golia, L.; Gardner, C.F., and Peric-Golia, M. (1979) Eur. J. Pharmacol. 55, 407-409.
4. Peric-Golia, L.; Gardner, C.F., and Peric-Golia, M. (1981) Endocrinology 108, A371.
5. Mahley, R.W.; Bennett, B.D.; Morre, D.J.; Gray, M.E.; Thistlethwaite, W., and LeQuire, V.S. (1971) Lab Invest 25, 435-444.
6. Miller, J.L., and Hendricks, M.S. (1981) Horm. Metabol. Res. 13, 506-507.
7. Peric-Golia, L.; Gardner, C.F., and Peric-Golia, M. (1980) Peptides 1, 381-382.
8. Weigensberg, B.J., and McMillan, G.C. (1959) Amer. J. Clin. Pathol. 31, 16-25.
9. Schwenk, E., and Werthessen, N.T. (1952) Arch. Biochem. Biophys. 40, 334-341.
10. Jung, D.H.; Biggs, H.G., and Moorhead, W.R. (1975) Clin. Chem. 21, 1526-1530.
11. Chung, B.H.; Wilkinson, T.; Geer, J.C., and Segrest, J.P. (1980) J. Lipid Res. 21, 284-291.
12. Touchstone, J.C.; Levitt, R.E.; Levin, S.S., and Soloway, R.D. (1980) Lipids 15, 386-387.
13. Raju, K., and Vijayan, E. (1981) Regulatory Peptides 2, 265-274.
14. Vijayan, E., and Raju, K. (1982) Experientia 38, 863-864.
15. Bissette, G.; Manberg, P.; Nemeroff, C.B., and Prange, A.J. Jr. (1978) Life Sci. 23, 2173-2182.
16. Klauda, H.G., and Zilversmit, D.B. (1974) J. Lipid Res. 15, 593-601.
17. Drevon, C.A.; Lilljenqvist, A.-C.; Schreiner, B., and Norum, K.R. (1979) Atherosclerosis 34, 207-219.
18. Green, P.H.R., and Glickman, M. (1981) J. Lipid Res. 22, 1153-1173.
19. Green, P.H.R.; Tall, A.R., and Glickman, R.M. (1978) J. Clin. Invest. 61, 528-534.
20. Rosell, S.; Rokaeus, A., and Theodorsson-Norheim, E. (1982) Scand. J. Gastroenterol. 18 (Suppl. 82), 59-67.
21. Chapman, J., and Mills, G.L. (1977) Biochem. J. 167, 9-21.
22. Peric-Golia, L., and Jones, R.S. (1960) Proc. Soc. Exp. Biol. Med. 105, 337-339.
23. Haslewood, G.A.D. (1967) Bile Salts, p. 61, Methuene and Co., Ltd., London.
24. Iga, T., and Klaassen, C.D. (1982) Biochem. Pharmacol. 31, 211-216.

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Autoxidation of *Acholeplasma laidlawii* Membranes

GUEY-SHUANG WU,[a] ROBERT A. STEIN,[a] JAMES F. MEAD[a,b]* and RONALD N. MCELHANEY,[c] [a]*Laboratory of Biomedical and Environmental Sciences, University of California, 900 Veteran Avenue, Los Angeles, CA 90024,* [b]*Department of Biological Chemistry, UCLA School of Medicine, Los Angeles, CA 90024, and* [c]*Department of Biochemistry, The University of Alberta, Edmonton, Alberta, Canada T6G 247*

ABSTRACT

Autoxidation of *Acholeplasma laidlawii* membranes (with equimolar ratio of palmitic and linoleic acid) lacks an obvious induction period, and the overall rate of disappearance of substrate does not follow closely that of typical autocatalytic kinetics. Throughout the course of autoxidation, the major oxygenated products isolated were hydroperoxides (as hydroxy esters) and compounds that gave rise to trihydroxy esters. The yield of trihydroxy esters was appreciable even at the early stage of the oxidation and eventually grew to surpass that of hydroperoxides. The positions of the three hydroxyl groups in the trihydroxy esters were determined to be mostly of the 1,2,5-type rather than 1,2,3-type arrangement. To a lesser extent, some degraded products, including dimethyl nonanedioate, methyl myristate, methyl pentadecanoate, methyl hexadecadienoate and methyl heptadecadienoate also were obtained. Dimethyl nonanedioate was a previously known degradation product from 9-hydroperoxide. The shorter chain esters presumably arise from the cleavage of α-hydroperoxides of palmitate and linoleate moieties.

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INTRODUCTION

Consequences of certain reactions occurring in organized assemblies such as monolayers and micelles have been demonstrated to be different from those in solution or neat systems (1). In an effort to understand the peroxidation reaction as it occurs in highly ordered biomembranes, a series of attempts has been made in our laboratory to study the reaction with several model systems (1-4). These model systems range from simple fatty acid monolayers to phospholipid liposomes of various constituents similar to those usually found in biomembranes. These studies have demonstrated that both the kinetics and product distribution vary in membrane-like arrangements. The kinetics are shifted from those of a classical autocatalytic reaction to an apparent first order reaction in some instances, and certain secondary products, which are only minor components in liquid phase, increase in organized systems. These changes appear to be brought about by two factors: the spatial arrangement of the lipid molecules and the fatty acid composition of the model membranes.

Past studies of lipid peroxidation in biomembranes have been concentrated on the different rates of reaction initiated by various agents. Little attention has been given to the nature of the products and their distribution. For example, the effect of chaotropic agents on the rate of peroxidation of submitochondrial particles and microsomes (5,6), of iron-ascorbate on rat tissue homogenates (7) and of copper sulfate on erythrocyte membranes (8), have been reported. The assessment of the extent of peroxidation in these studies has been either the measurement of malondialdehyde or diene conjugation produced, or physical parameters, such as permeability, viscosity and susceptibility to hemolysis.

In this study, we have used a simple, natural membrane, that of mycoplasma *Acholeplasma laidlawii* B *(A. laidlawii)*, to further investigate membrane peroxidation of model systems. The advantages of choosing *A. laidlawii* membranes are as follows: 1) the absence of cell walls or internal membranes makes the isolation of pure plasma membranes straightforward; 2) *A. laidlawii* strains do not require cholesterol or closely related sterols for growth, thus simplifying the membrane lipid constituents, and 3) the organism has a limited capability to synthesize or alter the endogenous or exogenous fatty acids. Therefore, in the presence of avidin to further suppress the biosynthetic capacity (9), the fatty acid composition of the membranes can be controlled entirely by the growth medium (10,11).

The consequences of membrane peroxidation reported here are mostly assessed from the viewpoint of the nature and distribution of the secondary products formed by the autoxidation. Attempts also are made to search for the possible routes of formation of these major products in biomembranes.

*To whom correspondence should be addressed.

EXPERIMENTAL PROCEDURES

Materials and Methods

The *A. laidlawii* strain B used in this study was grown in a lipid-poor growth medium supplemented with an exogenous fatty acid mixture containing 50 mole % each of linoleic and palmitic acids (12). Avidin was used to further suppress a small extent of endogenous fatty acid biosynthesis and exogenous fatty acid elongation during cell growth (13). Cells were harvested by centrifugation in the late log or early stationary phase of growth. The plasma membranes were isolated by osmotic lysis in either deionized water or phosphate buffer (14). The major lipids in *A. laidlawii* membranes have been found to consist of phosphatidylglycerol, monoglucosyldiglyceride, and glycerophosphoryl diglucosyldiglyceride (15).

Gas liquid chromatography (GLC) was carried out using a Hewlett Packard Gas Chromatograph Model 5830A. The columns used were a 0.20 × 300 cm coiled metal column packed with 10% SP2330 on 100/200 mesh Chromosorb WAW and a 0.20 × 180 cm glass column packed with 3% OV-101 on 100/120 mesh Gas Chrom Q. The trimethylsilyl derivatives were prepared by treating the hydroxy esters with TRI-SIL reagent (Pierce Chemical Co., Rockford, Illinois). The relative yields of the products were calculated as the area ratio of the product peak versus the methyl palmitate peak in the mixture. Thin layer chromatography (TLC) was carried out using precoated Silica Gel G plates (0.25 mm thick, Analtech, Inc., Newark, Delaware). Most of the products were separated with the solvent system, petroleum ether/diethyl ether/acetic acid (80:20:1 or 50:50:1, v/v/v), except trihydroxy esters, which were separated with the solvent system, chloroform/methanol (95:5, v/v). The spots were revealed by dipping the plates in a solution of 3% cupric acetate in 8.5% phosphoric acid and charring at 140 C. Mass spectroscopic analyses (GC/MS) were carried out on a Finnigan Model 300 quadrupole spectrometer coupled with a Varian Aerograph Series 1400 gas chromatograph. The GLC column used was a 0.20 × 180 cm metal coiled column packed with 3% OV-1 on 100/120 mesh Chromosorb WHP. High pressure liquid chromatography (HPLC) was performed on a Varian Liquid Chromatograph Model 8500 equipped with a Varian Vari-Chrom UV detector operating at 234 nm and a Hewlett Packard Model 3380A Integrator. The column was Partisil 10 (4.6 mm × 25 cm), and the solvent system for hydroxy esters was 2-propanol/hexane (0.75:100, v/v) with a flow of 80 ml/hr.

Autoxidation of *A. laidlawii* Membranes

About 5 ml of membrane suspension (in deionized water) containing 24 mg of lipid and 36 mg of protein was incubated at 40 C with shaking for the desired length of time. For the experiments with an initiator, 1 ml of 3 mM ascorbic acid (final concentration, 0.43 mM) and 1 ml of 1.5 mM $FeCl_3 \cdot 6H_2O$ (final concentration, 0.21 mM) were added to the suspension before incubation. After incubation, the membrane suspension was lyophilized to dryness. Extraction without prior lyophilization resulted in the loss of a considerable amount of glycolipid to the aqueous phase. The residue was extracted 3 times with chloroform/methanol/water (1:2:0.8, v/v/v), and the supernatant was removed after centrifugation (35,000 × g, 20 min). The solvent was removed from the combined extracts, and the dried residue was found to contain all of the lipid and some protein. A partition between aqueous methanol and chloroform phases at this step to remove protein was again found to result in loss of lipid; thus the protein was removed at the subsequent hydrolysis step. The residual lipid was hydrolyzed at 25 C with 4 ml of diethyl ether and 0.6 ml of KOH solution (3 g of KOH in 10 ml of methanol) (4). The isolated fatty acids were esterified with diazomethane, and the methyl esters were quantitated by GLC. The organism grown in a medium containing exactly 50 mole % of palmitic acid and linoleic acid was found to contain 47% palmitic acid and 52% linoleic acid in the isolated membrane fatty acids.

Preparation of 9,10-Dihydroxy-11-octadecenoic Acid, 11,12-Dihydroxy-9-octadecenoic Acid and 9,12-Dihydroxy-10-octadecenoic Acid

12-Hydroxy-9-*trans*-octadecenoic acid, which was prepared from commercial 12-hydroxy-9-*cis*-octadecenoic acid (16) for another purpose in this laboratory, was used to prepare *trans, trans*-9,11-octadecadienoic acid by thermal dehydration with subsequent collection of the product by distillation (17). The product was then purified by crystallization.

9,11-Octadecadienoic acid was epoxidized by an established procedure (18) with slight modification. 9,11-Octadecadienoic acid, 10 mg in 0.5 ml CH_2Cl_2, was mixed with 10 mg m-chloroperbenzoic acid in 0.5 ml CH_2Cl_2, and the mixture stirred at room temperature for 3 hr. The isolated epoxides can be hydrolyzed by perchloric acid in tetrahydrofuran/water to the desired dihydroxy acid. Alternatively, the epoxidation mixture of 9,11-octadecadienoic acid and m-chloroperbenzoic acid can be allowed to react overnight following which a

good yield of the diol mono-m-chlorobenzoate can be obtained. After hydrolysis and esterification with diazomethane, the positional isomers were separated by preparative TLC using petroleum ether/ether/acetic acid (50:50:1, v/v/v).

Ozonolysis of Hydroxy Unsaturated Esters

Samples to be ozonized were first acylated with an excess of trifluoroacetic anhydride in $CHCl_3$ to protect the hydroxyl groups. After removing the reagent and solvent under a stream of nitrogen, the samples, 0.1-1.0 mg in 1 ml CH_2Cl_2, were ozonized at Dry Ice/2-propanol temperature, and the ozonide reduced by addition of a 2-3-fold equivalent excess of triphenylphosphine (19). The reaction mixture was injected directly into the gas chromatograph. Samples were chromatographed on both a nonpolar, OV-101, and a polar, SP2330, column.

Preparation and Autoxidation of Linoleic Acid-9,11-Octadecadienoic Acid Monolayers

Pure linoleic acid, mixed with either 10% or 22% of 9,11-octadecadienoic acid, was used to form a monomolecular coating on Silica Gel G following the procedure described previously (1). The monolayers were incubated at 60 C for 3 hr. The fatty acids were recovered by extraction with methanol, and a mixture of KOH/methanol and ether was used to convert the peroxides to alcohols (4). The total crude product was analyzed by GLC.

RESULTS

The rate of autoxidation of *A. laidlawii* membranes (equimolar 18:2 and 16:0, with 18:2 exclusively at C-2 of the glycerol moiety) without an initiator is extremely slow. The disappearance of linoleic acid was about 20% after 126 hr of incubation at 40 C. As shown in Figure 1, a high level of conversion eventually was attained, after 17 days of incubation. A mild sonication of the membrane suspension accelerated the reaction considerably. The remaining linoleic acid was reduced to 46% after 138 hr of incubation. A plot of the logarithm of the remaining linoleic acid versus the length of incubation gave a reasonably straight line up to 126 hr. In contrast, the autoxidation of *A. laidlawii* membranes mixed with a low concentration of iron-ascorbate was faster; only 13% of the linoleic acid remained after 72 hr of incubation (Fig. 1).

Hydroperoxides, which were isolated and identified as hydroxy esters (4,20), were the major products at low conversion. With iron-ascorbate, the rate of production of hydroperoxides reached a maximum at about 40 hr and then fell rapidly with further incubation (Fig. 2). The 4 isomeric hydroxy esters, **1a**, **2a**, **3a** and **4a**, derived from the hydroperoxides, could be resolved completely by HPLC (4). The separation of 2 sets of geometrical isomers, **1a** + **3a** and **2a** + **4a**, also can be accomplished by GLC (peaks D, E and F, Fig. 3). The *trans, trans* isomers **2a** + **4a**, however, were found to be less stable than the corresponding *cis,trans* isomers **1a** + **3a**, at the GLC temperature. Therefore the ratio *cis,trans/trans,trans* obtained by GLC was always larger than that from HPLC. The relative yields of the four isomers obtained from HPLC are shown in Table 1 and

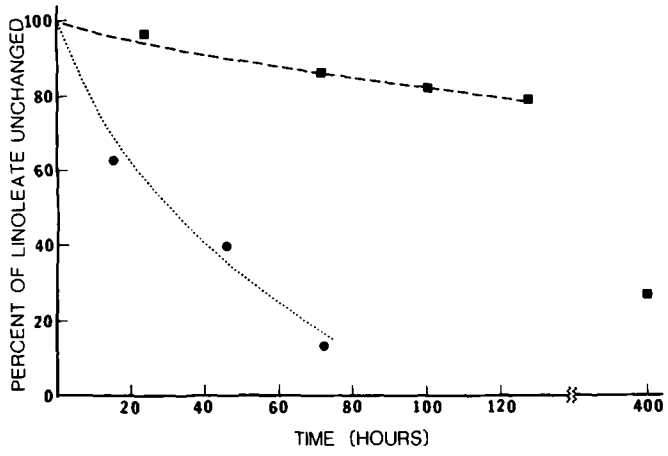


FIG. 1. Rate of autoxidation of linoleate in *A. laidlawii* membranes with and without added initiators. ----●----●----●----, with iron-ascorbate, and –■–■–■–, without iron-ascorbate.

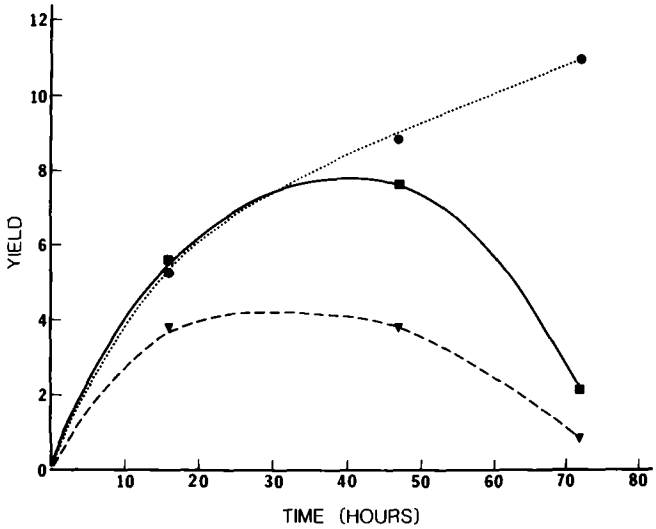


FIG. 2. Relative yields of 3 major products as a function of time in autoxidation of *A. laidlawii* membranes with iron-ascorbate. The yields were obtained from both HPLC and GLC. -▼ ▼ ▼, mixture of 1a and 3a; —■—■—■—, mixture of 2a and 4a, and ----●---●---●----, mixture of 5a, 6a, 7a and 8a.

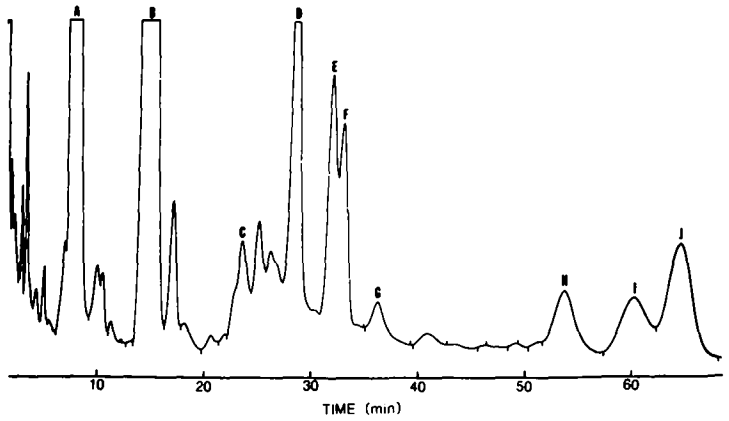


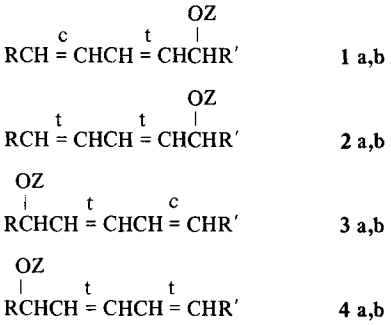
FIG. 3. Gas chromatograph of autoxidized products from *A. laidlawii* membranes. Membrane suspension was allowed to autoxidize for 47 hr at 40 C with iron/ascorbate, and after hydrolysis was analyzed as its trimethylsilyloxy ether, methyl ester derivatives. The separation was carried out on a 6 ft column packed with 3% OV-101 on Gas Chrom Q with temperature programming from 175 to 195 C at 0.8 C/min. A, methyl palmitate; B, methyl linoleate; C, 9,10- and 12,13-epoxyoctadecenoate; D, mixture of 1a and 3a; E and F, mixture of 2a and 4a; G, positional isomers of hydroxyepoxyoctadecenoate; H, mixture of 5a, 7a and unidentified products; I and J, mixture of 6a and 8a.

TABLE 1

Relative Yield of Isomers of Hydroxy Fatty Acids from *A. laidlawii* Membranes

Condition and length of incubation	1a	2a	3a	4a	1a + 3a/2a + 4a
+ Fe-ascorbate					
16 hr	0.20	0.27	0.21	0.31	0.70
47 hr	0.16	0.30	0.16	0.36	0.48
72 hr	0.033	0.090	0.032	0.101	0.34
– Fe-ascorbate					
99 hr	0.13	0.29	0.18	0.38	0.46

in Figure 2. In runs with iron-ascorbate, the ratio, *cis,trans/trans,trans* decreased consistently as the length of incubation increased, but without iron-ascorbate the ratio was smaller at the same extent of conversion.



1 a,b

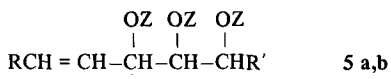
2 a,b

3 a,b

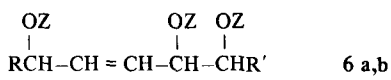
4 a,b

R = $CH_3(CH_2)_4$ –
R′ = $-(CH_2)_7COOCH_3$
a, Z = H
b, Z = TFA

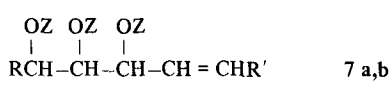
In addition to hydroperoxides, products that yield trihydroxy compounds were present in major quantity. The major pattern of product distribution was found to be essentially the same with or without added initiator. The yield of trihydroxy esters was appreciable early in the reaction and continued to increase to eventually surpass the yield of hydroperoxides (Fig. 2). The 4 isomers of trihydroxy esters, **5a**, **6a**, **7a** and **8a**, appeared as peaks H, I and J in the GLC of silylated crude autoxidation products (Fig. 3). Other products, such as hydroxyepoxides (peak G, Fig. 3) and dihydroxy esters, which appear in the region between peaks F and H, were present in minor quantities.



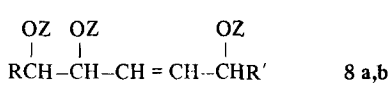
5 a,b



6 a,b



7 a,b



8 a,b

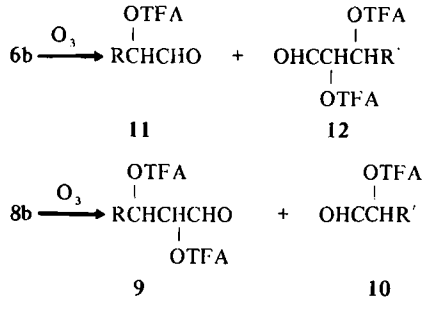
R = $CH_3(CH_2)_4$ –
R′ = $-(CH_2)_7COOCH_3$
a, Z = H
b, Z = TFA

In a liquid phase autoxidation of a mixture of approximately equimolar quantities of palmitic and linoleic acids, the isolated monomeric oxygenated product mixture was much more complex; in addition to trihydroxy esters, there were at least 5 other compounds produced in yields comparable to that of trihydroxy ester (4).

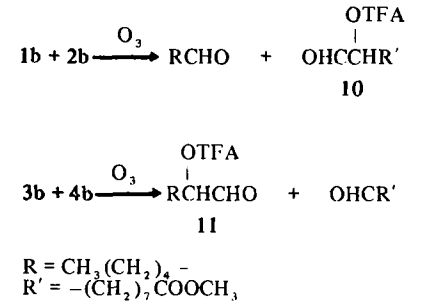
Since the autoxidation of *A. laidlawii* membranes produced a large quantity of product that yielded trihydroxy ester, attempts were made to establish unequivocally the positions of the 3 hydroxyl groups in this compound. The production of trihydroxy fatty esters from linoleate hydroperoxides has been reported previously (21,22). The GC/MS properties of trimethylsilyl derivatives of both 1,2,3- (**5a** and **7a**) and 1,2,5- (**6a** and **8a**) trihydroxy esters have been reported previously by this laboratory (4) and others (21,23). Compounds **5a** and **7a** give intense ions which are distinctive for the 1,2,3-substitution (e.g., ions 199 and 361 from **7a**). These ions result from the cleavage between two trimethylsilyloxy groups on C-11 and C-12. Using GC/MS alone, it was not possible to confirm the presence of **6a** and **8a** in a mixture, since all the major ions, 301, 259, 387 and 173, indicative of **6a** and **8a**, also are derived from **5a** and **7a**.

Therefore ozonolysis was used to establish the positions of 3 hydroxyl groups unequivocally. Relatively pure samples of trihydroxy esters were isolated by preparative TLC prior to ozonolysis. Isomers of trihydroxy esters (peaks H, I and J in Fig. 3) can be separated from most of the other products and also resolved partially from each other by TLC. The R_f values for peaks H, I and J are 4.4, 2.1 and 1.6 respectively. The spot with R_f 4.4 was found to contain only about 70% of peak H. The spots with R_f 2.1 and 1.6 were exclusively trihydroxy compounds represented by peaks I and J (Fig. 3) with the faster and slower compounds being I and J, respectively. The 2 peaks, however, could not be completely resolved by varying the solvent systems.

For the purpose of protecting the hydroxyl groups in the resulting hydroxyaldehyde and the ease of chromatographing the hydroxy compounds, **5a**, **6a**, **7a** and **8a** were converted to trifluoroacetates before ozonolysis. The ozonolysis of the mixture represented by peaks I and J (Fig. 3) gave 4 degradation products, **9**, **10**, **11** and **12**, as depicted in Scheme 1. The identification of these 4 products was carried out by comparison with the authentic compounds prepared by 2 independent routes. In the first route, the authentic compounds, **10** and **11**, were obtained from the ozonolysis of pure positional isomers of hydroxyoctadecadienoate (**1b** – **4b**). As shown in Scheme 2, trifluoroacetate **1b** + **2b** gave hexanal and the



SCHEME 1



SCHEME 2

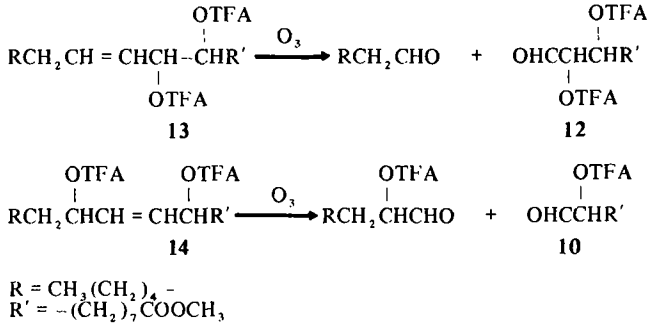
desired hydroxyaldehyde ester **10**. Correspondingly, **3b** and **4b** gave hydroxyaldehyde **11** and 9-oxononanoate. The identities of hexanal and 9-oxononanoate were confirmed by comparison with the ozonolysis products from pure methyl linoleate. From the ozonolysis of **1b − 4b**, the desired products were obtained. However, the ozonolysis was complicated by a rearrangement between the positional isomers. Starting with an HPLC pure sample of **1b + 2b**, some contamination of ozonolysis product from **3b + 4b** and vice versa always can be expected.

In the second attempt, the starting compounds were chosen so as to result in a more straightforward reaction at the ozonolysis step. Reaction of 9,11-octadecadienoic acid with m-chloroperbenzoic acid resulted in both epoxidation and the subsequent ring opening reaction to form the benzoate of the corresponding dihydroxy compounds. Hydrolysis of the benzoate gave 9,10-, 11,12- and 9,12-dihydroxyoctadecenoate. This mixture can be successfully separated by preparative TLC. The relative quantities of 9,10- and 11,12-dihydroxyoctadecenoate in a particular sample can be monitored by GC/MS, measuring the relative intensities of ions 259 and 285, representing fragments $CH(OTMS)(CH_2)_7COOCH_3$ and $CH(OTMS)CH=CH(CH_2)_7COOCH_3$, respectively.

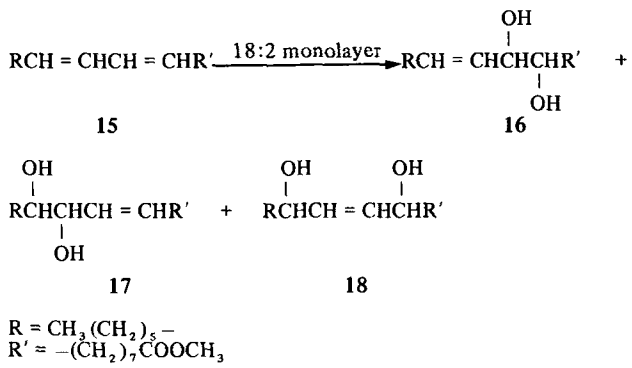
As indicated in Scheme 3, ozonolysis of **13** gave in addition to heptanal, dihydroxyaldehyde **12**, which coincides with the products from trihydroxy esters **6b + 8b** in GLC retention times in both polar and non-polar columns. Correspondingly, ozonolysis of **14** gave 2 degradation products. The higher molecular weight fragment matches that from ozonolysis of trihydroxy esters **6b + 8b** on both polar and non-polar columns.

The TLC isolated fraction, corresponding mostly to peak H (Fig. 3), gave ions 199, 361, 271, 275 and 285, which are characteristic of 1,2,3-trihydroxy esters, **5a** and **7a** (4). This fraction, when subjected to ozonolysis, gave, among other degradation products, a high yield of 9-oxononanoate, indicative particularly of structure **7b**. The minor component in this fraction, based on GC/MS, appears to be a methoxy-containing compound.

In a search for evidence to support an addition mechanism for the formation of trihydroxy esters, experiments were designed to see whether the supply of conjugated dienes in the autoxidation of linoleate enhances the production of addition products. In silica gel-sup-



SCHEME 3



SCHEME 4

ported pure linoleic acid monolayers (1), autoxidation produced only a minor quantity of dihydroxy esters. When **15** was incorporated into linoleic acid monolayers, a large quantity of dihydroxy esters **16**, **17** and **18** was produced upon autoxidation (Scheme 4). The dihydroxy esters obtained from linoleic acid-9,11-octadecadienoic acid monolayers were identified by comparing their behavior on GLC and TLC with those of authentic samples prepared by epoxidation of **15** followed by ring opening. The relative yields of **16**, **17** and **18** from linoleic acid-9,11-octadecadienoic acid monolayers are 2:2:7 respectively. The total yield of **16**, **17** and **18** obtained from the monolayers is proportional to the quantities of **15** included in the monolayers.

In the autoxidation of *A. laidlawii* membranes, in addition to the major products, some compounds appearing earlier than methyl palmitate on GLC also were noted (Fig. 4). The major cleavage product appears to be dimethyl nonanedioate (peak A, Fig. 4), presumably arising from the oxidation of methyl 9-oxononanoate. Other degradation products were found to be methyl myristate (peak B, Fig. 4), methyl pentadecanoate (peak C, Fig. 4), methyl hexadecadienoate (peak D, Fig. 4) and methyl heptadecadienoate (peak F, Fig. 4). These products were identified by the retention times on both polar and non-polar columns on GLC before and after hydrogenation, and also by the mass and fragmentation patterns obtained from GC/MS. The mass spec-

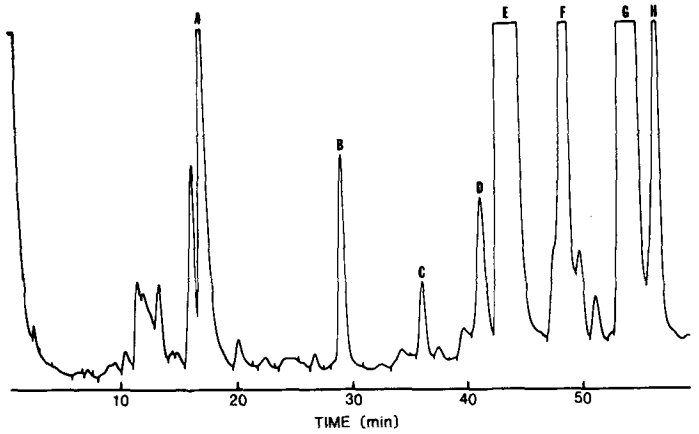


FIG. 4. Gas chromatogram of autoxidized degradation products from *A. laidlawii* membranes. The membrane suspension was allowed to autoxidize for 47 hr at 40 C with iron/ascorbate, and after hydrolysis was analyzed as methyl esters. The separation was carried out on a 6 ft column packed with 3% OV-101 on Gas Chrom Q with temperature programming from 105 to 190 C at 1.30 C/min. A, dimethyl nonanedioate; B, methyl myristate; C, methyl pentadecanoate; D, methyl hexadecadienoate; E, methyl palmitate; F, methyl heptadecadienoate; G, methyl linoleate and H, methyl stearate.

trometry of saturated, unsaturated and long chain diesters has been well established in the past (24,25).

DISCUSSION

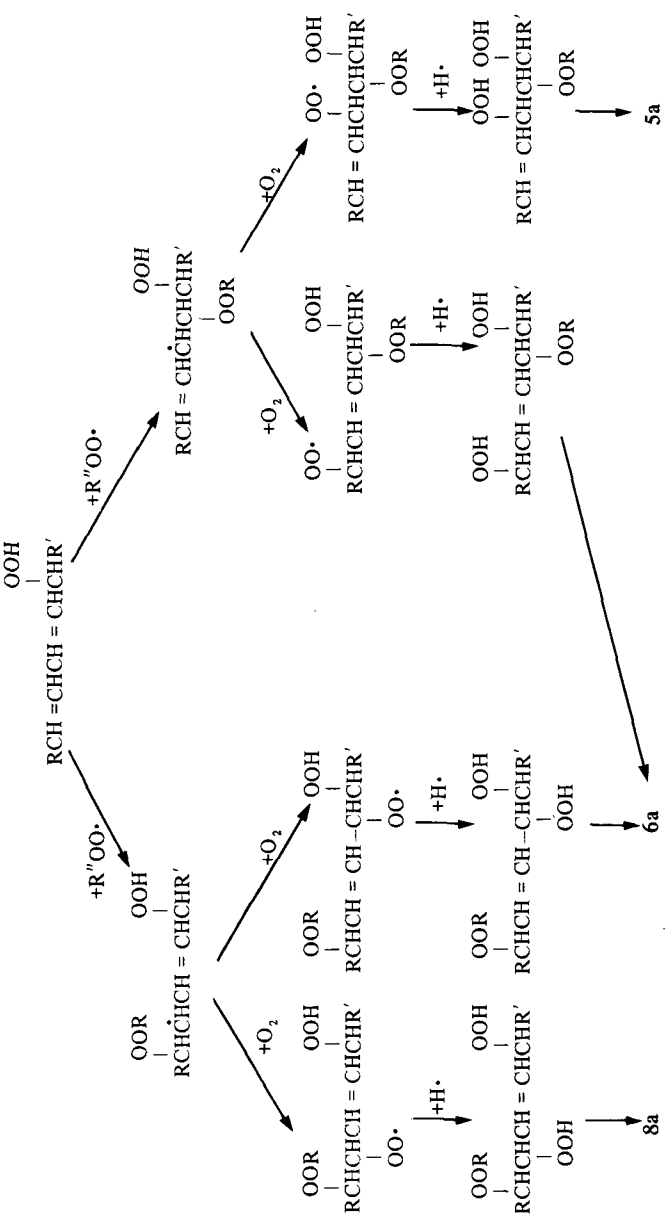
A. laidlawii membranes, with the advantages of simplicity in membrane isolation and manipulatable fatty acid composition, have been chosen frequently for studies of membrane properties. The autoxidation of *A. laidlawii* membranes without initiator proceeds extremely slowly at 40 C. The rate was accelerated considerably by the addition of a small amount of iron-ascorbate. The kinetics of the oxidation appear not to conform entirely to a typical autocatalytic reaction. The early phases indicated apparent first order kinetics, which lasted 10 hr with added initiator and 130 hr without.

The autoxidation of *A. laidlawii* membranes, with or without the addition of iron-ascorbate, up to the conversion of about 60%, gave hydroperoxides as major products. At a higher conversion, the product that yields trihydroxy esters dominates (Figs. 2 and 3). The ratio of *cis,trans/trans,trans* hydroperoxide isomers (Table 1) is a measure of the relative extent of 2 competing reactions for the peroxyl radicals: 1) abstraction of hydrogen atoms by the peroxyl radicals from neighboring molecules that leads to *cis,trans* isomer formation, and 2) β-scission to regenerate the pentadienyl system to yield *trans,trans* isomer (26). We previously reported that pure soybean phosphatidylcholine (PC) liposomes gave a considerably higher *cis,trans/trans,trans* hydroperoxide ratio (1.06) than that from neat linoleic acid (0.55) (4). In addition, the hydrogen abstraction process in pure soybean PC liposomes was suppressed considerably by the dilution of unsaturated moieties with saturated moieties, as in the case of 1:4 soybean PC-dipalmitoyl PC liposomes (the *cis,trans/trans,trans* ratio dropped from 1.06 to 0.28 [4]). This suggests that hydrogen abstraction is favored over β-scission not only by the hydrogen donating ability of hydrogens attached to bis-allylic carbons, but also by their accessibility because of the packing and orientation of the acyl chains. In *A. laidlawii* membranes, the *cis,trans/trans,trans* product ratio at the early stage represents a middle value (0.70) (Table 1) between pure soybean PC and soybean PC-dipalmitoyl PC liposomes. The difference in the 2 systems can be explained on the basis of linoleate concentration and its spatial arrangement in the membrane lipids. The proportion of *cis,trans* isomer also was found to decrease in *A. laidlawii* membranes as the incubation progressed. This reduction in *cis,trans/trans,trans* ratio could result from the decrease in readily accessible hydrogen donors or isomerization of *cis,trans* hydroperoxide to the *trans,trans* isomer (27,28). The ratio of *cis,trans/trans,trans* isomers from the run without iron-ascorbate was slightly higher than that from the run with this catalyst (0.46 and 0.34 respectively, Table 1) when compared at similar extended incubation periods. The ferric ion-assisted cleavage of hydroperoxide to peroxyl radical might increase the proportion of *trans,trans* isomer in the mixture and thus lower the *cis,trans/trans,trans* ratio. However, in the system with excess reducing agent, the contribution from ferric ion is unknown; therefore, the role played by iron in this aspect is not clear.

The trihydroxy esters **5a**, **6a**, **7a** and **8a** are the major secondary products in the autoxidation of *A. laidlawii* membranes with or without initiators throughout the incubation period. As shown in Figure 2, the yields of trihydroxy esters are as great as those of *trans,trans* hydroxy esters (derived from the corresponding hydroperoxide) even at the early stage of autoxidation.

It has been reported that in the enzymatic (21,23,29) and iron-catalyzed (22) conversion of hydroperoxides, the trihydroxy esters obtained were exclusively a mixture of **6a** and **8a** based on the results from GC/MS and periodic acid oxidation. In the present case, both 1,2,3- (**5a** and **7a**) and 1,2,5- (**6a** and **8a**) trihydroxy esters were found in the autoxidation of *A. laidlawii* membranes, with the ratio of **6a** + **8a**/**5a** + **7a** equal to approximately 5 at any stage of autoxidation.

The mechanism of formation of trihydroxy esters from the autoxidation of linoleate or from the preformed hydroperoxide has been dealt with in detail in the past in only one instance, in which it was suggested briefly to arise from either 1,2- or 1,4- addition of hydorxyl radicals to the conjugated diene system of the 9- and 13-dienol intermediates or from trihydroperoxides (30). In the autoxidation of *A. laidlawii* membranes, an addition mechanism for the formation of trihydroxy esters depicted in Scheme 5 accounts for the existing experimental evidence. A sequence of reactions leading to the trihydroxy esters originates from addition of a peroxyl radical to either end of the conjugated system of a hydroperoxide. The resulting allylic radical is oxygenated to form a triperoxide which is reduced during isolation to the triol. An epoxide ring opening cannot be ruled out as contributing to the polyol prod-


RCH =CHCH = CHCHR′
+R″OO·
+R″OO·
RCHCHCH = CHCHR′
RCH = CHCHCHCHR′
+O2
+O2
+O2
+O2
RCHCHCH = CHCHR′
RCHCH = CH–CHCHR′
RCHCH = CH–CHCHR′
RCH = CHCHCHCHR′
RCHCH = CHCHCHR′
RCH = CHCHCHCHR′
+H·
+H·
+H·
+H·
8a
6a
5a
R = CH3(CH2)4–
R′ = –(CH2)7COOCH3
R″ = any alkyl

SCHEME 5

ucts. At low pH, linoleate hydroperoxide has been reported to give trihydroxy compounds by way of an epoxide intermediate (H. W. Gardner, private communication). The relative amounts of trihydroxy compounds that should be ascribed to the various possible schemes have not been determined.

There are several lines of evidence that support the feasibility of a peroxyl radical addition to conjugated diene of a hydroperoxide for the formation of trihydroxy esters. A mixture of 9,10-epoxy-11-hydroxy-12-octadecenoate and 11-hydroxy-12,13-epoxy-9-octadecenoate obtained from the autoxidation of linoleic acid monolayers (1,2), when subjected to the conditions of autoxidation of *A. laidlawii* membranes, gave no trihydroxy esters. This observation excludes the possibility that the trihydroxy esters are the ring opening products of these particular hydroxyepoxy compounds.

An experiment also was performed to measure the extent of addition of peroxyl radicals to the conjugated diene. In silica gel-supported linoleic acid monolayers (1), different proportions of 9,11-octadecadienoic acid were incorporated. The autoxidation of the mixed monolayers gave, in addition to the expected products from linoleic acid, a large quantity of a mixture of 9,10-, 9,12- and 11,12-dihydroxyoctadecenoic acid, presumably formed by the addition of peroxyl radicals to the conjugated acid (Scheme 4). On GLC, dihydroxy esters were increased considerably in the samples after an alkaline hydrolysis (20). The ratio of 1,4-diol to 1,2-diol in these reactions was approximately 2 to 1. The predominance of the 1,4-addition was often noted for the addition of radicals to the conjugated systems (31). The yields of dihydroxy esters were found to be proportional to the amount of 9,11-octadecadienoic acid included in the linoleic acid monolayers. Without 9,11-octadecadienoic acid, there were no appreciable amounts of dihydroxy esters detected from the autoxidation of linoleic acid monolayers.

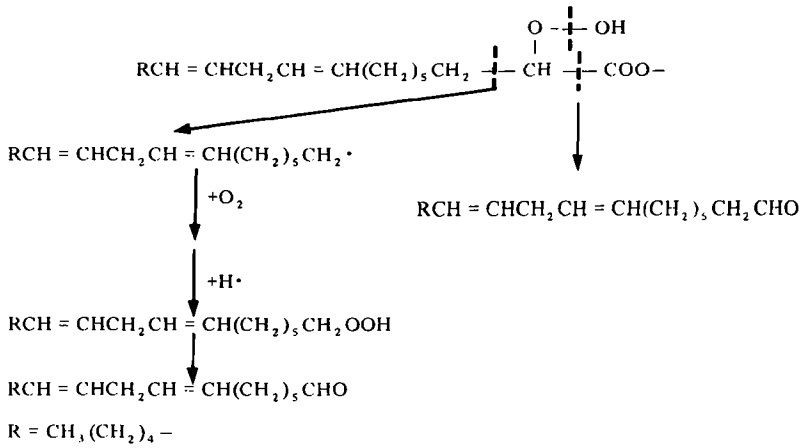
The degradation of hydroperoxides through the formation of alkoxyl radical has been regarded as one of their major secondary reactions. In the thermal decomposition of methyl linoleate hydroperoxide, all of the possible β-scission products of oxy radicals, except those resulting in vinyl radicals, have been isolated (32,33,34). It also has been reported that a normal spontaneous scission favors the cleavage of the carbon linkage between the double bond and the alkoxyl radical. However, under conditions such as high temperature, high pH or metal catalysis, a larger proportion of other cleavage products also was found (35). In the *A. laidlawaii* membrane system, following a prolonged incubation at 40 C, only 1,9-nonandioate was detected in a major quantity. The results seem to indicate the predominance of a β-scission between the carbon of alkoxyl radical and the neighboring double bond. Since the major product is the same with or without the initiator, the site of the cleavage apparently is not affected by the presence of iron. Hexanal or hexanoic acid from the methyl terminal end of linoleic acid probably was lost prior to GLC. The predominance of 1,9-nonanedioate over other cleavage products also was apparent in the autoxidation of soybean PC liposomes (Wu, G.-S., unpublished observations).

In addition to the β-scission products of 9- and 13-oxy radicals, a different group of fragmentation products with yields comparable to those of 1,9-nonanedioate also was obtained from *A. laidlawii* membranes (Fig. 4). Among these products, heptadecadienoate and hexadecadienoate appear to arise from carbon-carbon scission on either side of the α-hydroperoxide derived from the oxygenation of the α-carbon radical of linoleate. The shorter chain esters were obtained either directly from the oxidation of the corresponding aldehyde or through prior oxygenation and hydrogen atom abstraction as depicted in Scheme 6. Similarly, pentadecanoate and hexadecanoate probably were derived from the palmitate. Evidence for the possible precursor, α-hydroperoxyhexadecanoate, was established by detecting in small yield, α-hydroxyhexadecanoate. The presence of α-hydroperoxyoctadecadienoate was not established, since the expected retention time of α-hydroxyoctadecadienoate was similar to that of **1a** + **3a**.

The cleavage product from α-alkoxyl radicals was detected, although to a lesser extent, among the products of autoxidation of soybean PC liposomes, but not from the liquid phase oxidation of a linoleate-palmitate mixture of the same composition. It also was found recently in a prolonged incubation of human erythrocyte ghosts (36).

In the autoxidation of *A. laidlawii* membranes, there are 2 important products that are either absent or in much smaller proportion in liquid phase or in solution (4,22). These are the trihydroxy esters, particularly **6a** and **8a**, and the degradation products originating from an α-attack on fatty acid chains.

The organized semirigid assemblies, such as monolayer films, micelles and artificial bilayers have been known to divert the course of a chemical process and result in consequences that are different from those found in liquid



SCHEME 6

phase or in solution. The control of the fate of peroxyl radicals to favor the addition to the conjugated systems rather than other courses in the membranes might be effected by 2 factors which are specific for these systems. 1) Because of a considerably higher microviscosity, the radical escape efficiencies are significantly smaller than in the liquid phase or in solution (37,38). Therefore, the peroxyl radicals, once formed, are slow to diffuse away and remain in the vicinity of the conjugated hydroperoxides. 2) In membranes, the closeness in packing and alignment of lipid molecules could be such as to facilitate the addition reaction.

The abstraction of α-hydrogens from fatty esters is a higher energy process than the removal of allylic hydrogens, and its cause in the *A. laidlawii* membranes is not clear. It could be facilitated by a relatively larger concentration of initiator at the hydrophilic surface in comparison with that in the hydrocarbon interior of the membrane. Presumably this is due to a limited diffusion of an initiator which is supplied through the aqueous medium (37).

ACKNOWLEDGMENTS

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REFERENCES

1. Wu, G.-S.; Stein, R.A., and Mead, J.F. (1977) Lipids 12, 971-978.
2. Wu, G.-S.; Stein, R.A., and Mead, J.F. (1978) Lipids 13, 517-524.
3. Wu, G.-S.; Stein, R.A., and Mead, J.F. (1979) Lipids 14, 644-650.
4. Wu, G.-S.; Stein, R.A., and Mead, J.F. (1982) Lipids 17, 403-413.
5. Hatefi, Y., and Hanstein, W.G. (1970) Arch. Biochem. Biophys. 138, 73-86.
6. Hanstein, Y., and Hatefi, W.G. (1970) Arch. Biochem. Biophys. 138, 87-95.
7. Barber, A.A. (1966) Lipids 1, 146-151.
8. Chan, P.C.; Peller, O.G., and Kesner, L. (1982) Lipids 17, 331-337.
9. Silvius, J.R., and McElhaney, R.N. (1978) Can. J. Biochem. 56, 462-469.
10. Razin, S.; Turtellotte, M.E.; McElhaney, R.N., and Pollack, J.D. (1966) J. Bacteriol. 91, 609-616.
11. Silvius, J.R.; Saito, Y., and McElhaney, R.N. (1977) Arch. Biochem. Biophys. 182, 455-464.
12. Saito, Y., and McElhaney, R.N. (1977) J. Bacteriol. 132, 485-496.
13. Silvius, J.R., and McElhaney, R.N. (1978) Can. J. Biochem. 56, 462-469.
14. Razin, S. (1975) Prog. Surf. Membr. Sci. 9, 257-312.
15. Wieslander, A., and Rilfors, L. (1977) Biochim. Biophys. Acta 466, 336-346.
16. Krafft, F. (1888) Chem. Ber. 21, 2730-2737.
17. Mangold, C. (1894) Monatsh. 15, 307-315.
18. Fieser, L.F., and Fieser, M. (1967) Reagents for Organic Synthesis, Vol. 1, pp. 135-136, John Wiley and Sons, Inc., New York.
19. Stein, R.A., and Nicolaides, N. (1962) J. Lipid Res. 3, 476-478.
20. O'Brien, P.J. (1969) Can. J. Biochem. 47, 485-492.
21. Streckert, G., and Stan, H.J. (1975) Lipids 10, 847-854.
22. Gardner, H.W.; Kleiman, R., and Weisleder, D. (1974) Lipids 9, 696-706.
23. Graveland, A. (1970) J. Am. Oil Chem. Soc. 47, 352-361.
24. Sun, K.K., and Holman, R.T. (1968) J. Am. Oil Chem. Soc. 45, 810-817.
25. Gunstone, F.D. (1970) Topics in Lipid Chemistry, pp. 369-440, John Wiley and Sons, New York.
26. Porter, N.A.; Weber, B.A.; Weenen, H., and Khan, J.A. (1980) J. Am. Chem. Soc. 102, 5597-5601.
27. Chan, H.W.-S.; Costaras, C.T.; Prescott, F.A.A., and Swoboda, P.A.T. (1975) Biochem. Biophys.

Acta 398, 347-350.
28. Chan, H.W.-S.; Levett, G., and Matthew, J.A. (1979) Chem. Phys. Lipids 24, 245-256.
29. Galliard, T.; Phillips, D.R., and Matthew, J.A. (1975) Biochim. Biophys. Acta 409, 157-171.
30. Frankel, E.N.; Neff, W.E., and Rohwedder, W.K. (1977) Lipids 12, 908-913.
31. Walling, C., and Huyser, E.S. (1963) in Organic Reactions (Cope, A.C., ed.) Vol. 13, pp. 239-289, John Wiley and Sons, New York.
32. Chan, H.W.-S.; Prescott, F.A.A., and Swoboda, P.A.T. (1976) J. Am. Oil Chem. Soc. 53, 572-576.
33. Frankel, E.N.; Neff, W.E., and Selke, E. (1981) Lipids 16, 279-285.
34. Gardner, H.W.; Plattner, R.D., and Selke, E. (1983) J. Am. Oil Chem. Soc. 60, 716.
35. Kimoto, W.I., and Gaddis, A.M. (1969) J. Am. Oil Chem. Soc. 46, 403-408.
36. Bunick, F.J., and Nawar, W.W. (1983) J. Am. Oil Chem. Soc. 60, 716.
37. Winterle, J.S., and Mill, T. (1980) J. Am. Chem. Soc. 102, 6338-6339.
38. Turro, N.J., and Weed, G.C. (1983) J. Am. Chem. Soc. 105, 1861-1868.

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Phospholipid Studies of Marine Organisms: New Branched Fatty Acids From *Strongylophora durissima*

AMITAVA DASGUPTA, ESER AYANOGLU and **CARL DJERASSI,*** *Department of Chemistry, Stanford University, Stanford, CA 94305*

ABSTRACT

The phospholipids of the sponge *Strongylophora durissima* were analyzed. The major phospholipids present were phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylglycerol (PG) and phosphatidylinositol (PI). The major fatty acid components of the phospholipids consisted of short chain (C_{14}-C_{19}) and very long chain (C_{25}-C_{30}) "Demospongic" acids. Three novel branched Δ^5 monounsaturated acids, *Z*-19-methyl-5-pentacosenoic, *Z*-19-methyl-5-hexacosenoic and *Z*-19-methyl-5-heptacosenoic acids were encountered in the sponge. The 3-saturated counterparts of these compounds, 19-methylpentacosanoic, 19-methylhexacosanoic and 19-methylheptacosanoic acids, as well as 19-methyltetracosanoic and 20-methyloctacosanoic acids also are hitherto undescribed acids present in the sponge. Trace amounts of 2 very long chain acids also were detected and their structures tentatively assigned as 19,21-dimethylheptacosanoic and 20,22-dimethyloctacosanoic acids. The distribution of these fatty acids according to phospholipid head groups also was described.

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INTRODUCTION

The detection of novel sterols with unusual side chains and nuclei in marine invertebrates (1,2) and their possible role in the cell membrane structure drew our attention to the other major lipid membrane components, the phospholipids. Bergmann and Swift initially reported the presence of high amounts of C_{24}, C_{26}, C_{28} fatty acids in sponges (3). In the late seventies Litchfield and his co-workers also reported the presence of new fatty acids with very long chains (24 to 30 carbon atoms) from Demospongiae (4-7). The occurrence of long chain fatty acids and their possible role as constituents of membrane phospholipids in a number of sponges recently has been evaluated (8).

In connection with our research in the field of membrane phospholipid composition, we have concentrated on sponges in which "unusual" sterols are detected in large amounts and conventional sterols such as cholesterol are absent or present only in small amounts. Our studies already have revealed the presence of novel branched (9-11), cyclopropane (11) and methoxy (12,13) fatty acids with very long chains in the phospholipids of various members of Demospongiae. Quantitative analysis of phospholipids indicates that these acids are mostly accumulated in major amino groups containing phospholipids PE and PS. PC generally was found to be a minor component. Additionally, analysis of the sponge *Parasperella psila* as well as *Axinella verrucosa* led to the surprising observation that 5,9-hexacosadienoic acid ($\Delta^{5,9}$-26:2) is virtually the exclusive acid in the phospholipids with amino-containing head groups. This is unusual because conventional membrane phospholipids generally are known to contain 2 different fatty acyl fragments. Our experiments on the Pacific sponge *Parasperalla psila* (E. Ayanoglu, K. Kurtz and C. Djerassi, unpublished data) using centrifugation of enzymatically disaggregated cells on a stepwise gradient of Ficoll also indicate that PE and PS are enriched in denser layers, which contain no or only small amounts of bacterial cells, based on electron microscopic studies.

Almost all straight chain or methyl branched demospongic acids consist of a typical diunsaturation pattern ($\Delta^{5,9}$), first encountered by Litchfield et al. (4-7). We now wish to report the presence of a new group of demospongic acids with Δ^5 monounsaturation and with branching from the phospholipids of the sponge *Strongylophora durissima* which contains strongylosterol (14-16) as the major sterol and is almost devoid of cholesterol.

EXPERIMENTAL

Strongylophora durissima sponge colonies were collected at the Laing Island Marine Station near Madang, Papuea-New Guinea. Total phospholipids were extracted with chloroform/methanol (1:1, v/v) at 5 C as described earlier (9). The crude extract was purified by column chromatography using silicic acid (100 mesh) as stationary phase and chloroform/tetrachloromethane (2:1, v/v), acetone and methanol re-

*To whom correspondence should be addressed.

spectively as mobile phase (17). The methanol fraction contained the phospholipids. The extract was kept under argon or nitrogen at −10 C containing 0.002% BHT as preservative. Commercially prepared precoated silicagel thin layer chromatography (TLC) plates (0.2 mm thickness) were used for all TLC analyses. Molybdenum Blue was used as a general spray reagent for all phospholipids. Other spray reagents employed for identification of phospholipid head groups include: Ninhydrin (PE, PS), Dragendorff (PC), and Periodate-Schiff (DPG, PG, PI). Rhodamine 6G was used as a non-destructive spray reagent for visualizing fatty acid methyl esters, pyrrolidides, and different phospholipid bands. Quantitative estimation of different classes of phospholipids was done by analyzing the phosphate content of different bands by a spectrophotometric assay using Fiske and Subbarow Reagent (18-19). The developing solvents for phospholipids were $CHCl_3$/MeOH/H_2O (65:30:5, v/v) and $CHCl_3$/MeOH/AcOH/H_2O (50:30:4:2, v/v). The latter solvent was used for the quantitative estimation of the head group phosphates. The developing solvents for fatty acid methyl esters were hexane/ether (80:20, v/v) and for pyrrolidides hexane/ether (20:80, v/v).

The capillary gas chromatography (GC) analyses were carried out by using a Carlo Erba series 4160 Fractovap chromatograph equipped with a fused silica column (30 mm × 0.32 mm) coated with SE-54 (J & W Scientific Inc.), a model 400 LT programmer, and a flame ionization detector (FID). The initial oven temperatures were 70 and 130 C and the final oven temperature was 290 C, with gradients of 5 C/min or 10 C/min for the analysis of fatty acid methyl esters or pyrrolidides.

Either a Finnigan MAT-44 GC/MS system carrying spiral glass column (1.80 m × 2.0 mm) coated with 3% OV-17 on GCQ or a Ribermag R10-10 quadrupole mass spectrometer with a Carlo Erba series 4160 Fractovap chromatograph containing a fused silica column (28 m × 0.32 mm) with SE-54 (J & W Scientific) were used for capillary gas chromatography-mass spectrometry (GC/MS) analysis of phospholipid fatty acids.

^{1}H-NMR spectra were run on a Varian Associates HA-100 NMR instrument. A Nicolet 7000 series FT-IR was used for recording infrared spectra.

The total fatty acid methyl esters were prepared by treating the dry phospholipid extract with 1.25 N HCl in methanol for 30 min under reflux (20). The fatty acid contents of individual head groups were analyzed by separating individual phospholipid classes by preparative TLC and transesterification of each class by the use of 14% boron trifluoride in methanol (10 min, 100 C) (21). N-Acyl-pyrrolidide derivatives were prepared by treating the methyl esters with pyrrolidine/acetic acid (10:1, v/v) in a screw capped vial (1 hr, 100 C) followed by extraction with ether and purification by preparative TLC. Hydrogenation of fatty acid methyl esters was carried out by stirring the mixture in methanol under a balloon filled with hydrogen using platinum (IV) oxide as catalyst.

The high performance liquid chromatography (HPLC) separation of fatty acid methyl esters was achieved by using a 50 cm × 9 mm Whatman ODS-2 reversed phase column, a Waters M-6000 A pump, a Valco loop injector and a Waters R 401 refractometer detector. The eluting solvent was absolute methanol.

The degradation studies for the location of double bonds were carried out by $NaIO_4$/$KMnO_4$ oxidative degradation in t-butanol following esterification of the resulting carboxylic acid with 1.25 N HCl in methanol (22).

Two methyl branched esters, methyl 14,18-dimethylnonadecanoate (7) and methyl 14-methyleicosanoate (16) were synthesized as shown in Figure 1 for comparison with the degradation compound obtained from the monoenoic C_{26} acid methyl ester derived from the sponge. 3,7-Dimethyl-6-octenal (2) was obtained by the oxidation of 3,7-dimethyl-6-octanol (1) with pyridinium dichromate in dry dichloromethane (93% yield). 11-Undecyltriphenylphosphonium bromide (4) was prepared from 11-bromoundecanoic acid (3) and triphenyl phosphine in refluxing benzene (overnight). The Wittig reaction of 2 with 11-undecyltriphenylphosphoranylidene in tetrahydrofuran/dimethylsulfoxide (1:1, v/v) at room temperature (2 hr) gave 14,18-dimethyl-11,17-nonadienoic acid (5) in 20% yield. 11-Undecyltriphenylphosphonium bromide was deprotonated at 0 C with n-butyllithium. The unsaturated acid 5 was converted to the corresponding methyl ester (6) with diazomethane and hydrogenated with platinum (IV) oxide in methanol at room temperature for 8 hr to provide methyl 14,18-dimethylnonadecanoate (7).

3-Methylnonanoic acid (10) was prepared from 1-bromohexane (8) through a 1,4 ring-opening addition to β-butyrolactone (9) by a Grignard reaction in tetrahydrofuran/methyl sulfide (20:1, v/v) in the presence of copper (I) iodide (50% yield). The acid was converted to its methyl ester by diazomethane. The corresponding aldehyde (13) was obtained by the use of $LiAlH_4$ in ether (room temp., 2 hr) followed by oxidation with pyridinium chloro-

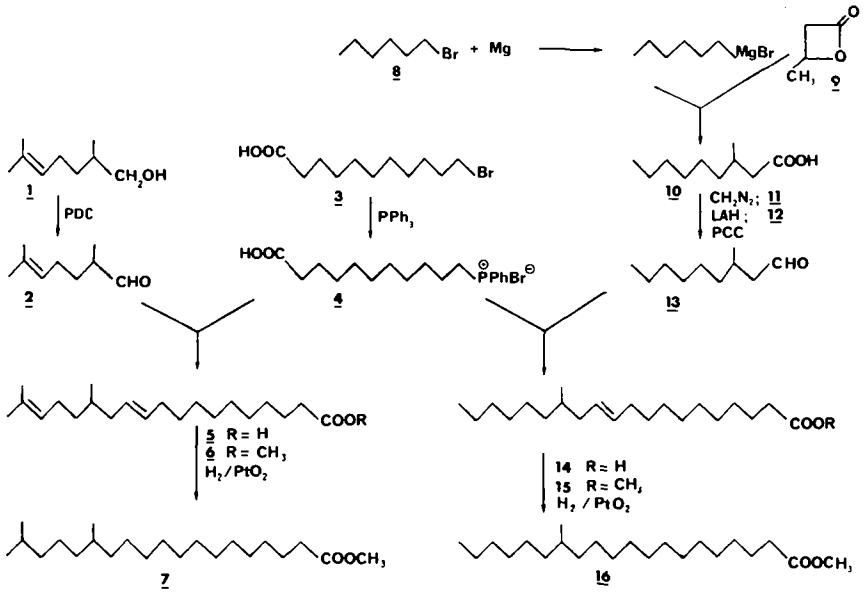


FIG. 1. The synthetic routes to methyl 14,18-dimethylnonadocanoate (7) and to methyl 14-methyleicosanoate (16).

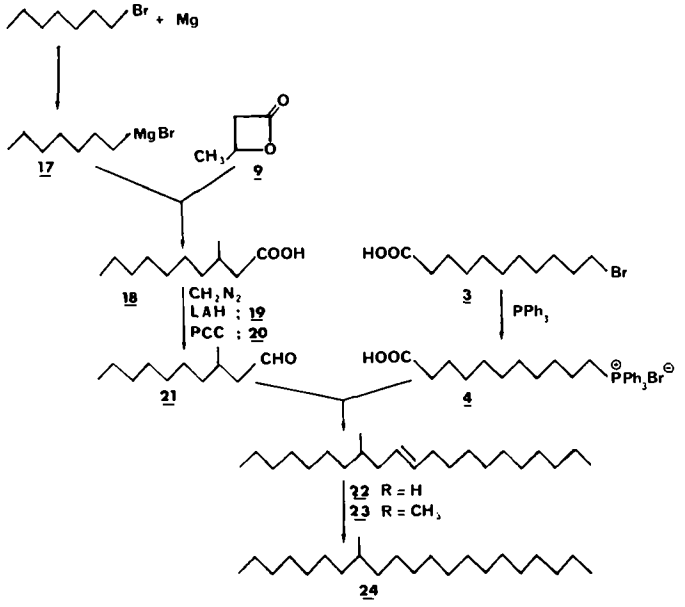


FIG. 2. The synthesis of methyl 14-methylheneicosanoate.

chromate in dichloromethane (room temp., 2 hr). The Wittig reaction of 13 with 11-undecyltriphenylphosphonium bromide produced the monounsaturated compound 14-methyl-11-eicosenoic acid (14) (yield 16%). Its methyl ester (15) was hydrogenated in the usual way to obtain methyl 14-methyleicosanoate (16).

Methyl 14-methylheneicosanoate (24) was synthesized as shown in Figure 2 for comparison with the degradation compound obtained from the monoenoic C_{27} acid methyl ester. For this purpose heptylmagnesium bromide (17) was treated with β-butyrolactone (9) in the presence of copper (I) iodide to give 3-methyldecanoic acid 18. Its corresponding aldehyde (21) was obtained following the above-men-

TABLE 1

The Major Phospholipids of *Strongylophora durissima*

Phospholipid class	Mol %[a]
Phosphatidylethanolamine (PE)	21 ± 1.2
Phosphatidylglycerol (PG)	14 ± 1.7
Phosphatidylcholine (PC)	21 ± 1.0
Phosphatidylserine (PS)/ Phosphatidylinositol (PI)[b]	24 ± 1.3

[a]Average of 4 replicates; percentages are based on relative phosphate content.

[b]PI comprised a small portion, less than 5% of the total mixture.

tioned procedure. The Wittig reaction of 21 with 4 followed by methyl esterification and hydrogenation in the usual manner afforded methyl 14-methylheneicosanoate (24). Synthetic reaction products and intermediates were characterized by their proton nuclear magnetic resonance (^{1}H-NMR), infrared (IR) and MS spectral data.

RESULTS AND DISCUSSION

Quantitative estimation of the phospholipids using solvent elution and spectrometric analysis of each TLC spot indicated the presence of 5 major phospholipid head groups, namely PE, PG, PC, PI and PS. The percentage of these major phospholipid head groups was calculated from 4 replicates and the results are summarized in Table 1. The capillary GC analysis of total fatty acid methyl esters from the sponge *Strongylophora durissima* indicated the presence of approximately 60 peaks out of which 27 peaks contribute to 92% of the total fatty acid methyl ester mixture. Many peaks, especially the conventional ones, were usually identified by comparison of the mass spectra and equivalent chain length (ECL) values (23) of the known standards. A capillary GC analysis of the hydrogenated derivatives of total fatty acids also was useful for identification. The pyrrolidides of the total fatty acids and hydrogenated derivatives also were analyzed by GC/MS. In general, a 12 atomic mass unit (amu) difference between the 2 most intense peaks of fragments containing n and n − 1 carbon atoms in the acid moiety indicates a double bond between n and n + 1 carbon in a pyrrolidide derivative (24). A peak of reduced intensity belonging to a carbon atom surrounded by 2 enhanced peaks shows the presence of methyl branching at that carbon atom of the molecule.

Taking an example from Table 2, the pyrrolidide of the monounsaturated and branched acid 14 (methyl ester M^+ 408, hydrogenated methyl ester M^+ 410) in our mixture showed a molecular ion peak corresponding to a C-26 acid (M^+ 447); and a 12 amu difference between C_4 (m/z 140) and C_5 (m/z 152) (Fig. 3A) indicating the presence of a double bond at C-5. In addition, a peak with reduced intensity at C_{19} (m/z 348) with concurrent enhanced C_{18} and C_{20} fragment peaks (m/z 334 and m/z 362) clearly disclosed a branching at C_{19}. A slightly diminished fragment peak at C_{24} (m/z 418) indicated the possibility of an iso-branching. Hydrogenation of this compound yielded the naturally occurring (Table 2) acid 15 whereupon the diminished fragment peak at C_{19} shifted to m/z 350. A slightly reduced fragment peak at m/z 420 also was observed (Fig. 3B). The monounsaturated acid 14 exhibited a notable absence of absorptions in the 980-968 cm^{-1} region of its infrared spectrum, indicating the presence of a *cis* rather than *trans* double bond (9,25). The position of this double bond at C_5 also was confirmed by oxidative degradation which gave a C_{21} acid (methyl ester M^+ 340) as the monofunctional reaction product. The pyrrolidide of this degradation product showed a diminished peak at C_{14} (m/z 280) with the expected enhanced fragment peaks at m/z 264 (Fig. 4A) and m/z 294. A slightly diminished fragment peak at C_{18} (m/z 350) again raised the possibility of an iso-branching (24).

In order to make an unambiguous assignment of the structure of 14, the 2 possible degradation products 14-methyleicosanoic (16) and 14,18-dimethylnonadecanoic (7) acids were synthesized as shown in Figure 1. The mass spectrum of the pyrrolidide derivative of 14,18-dimethylnonadecanoic acid (7) was similar to the pyrrolidide of the natural degradation product, except that the fragment peak at m/z 350 was more visibly diminished in the case of the synthetic compound (Fig. 4A and C). More significantly, the methyl ester of 14,18-dimethylnonadecanoic acid (7) gave a slightly shorter retention time than that of the natural product, thus demonstrating that they could not be identical.

On the other hand, 14-methyleicosanoic acid (16) had an identical retention time with the natural product as evidenced by the coinjection in the capillary GC. The mass spectra of their pyrrolidide derivatives also were identical (Fig. 4A and B). The fragment peaks at m/z 350 were slightly diminished in both cases. Based on these observations, the structure of the degradation product had to be 14-methyleicosanoic acid whereupon it follows that natural compound is 19-methyl-5-pentacosenoic acid (14 in Table 2). The only unanswered question is the possible chiral nature and

TABLE 2

Identified Major Fatty Acids from the Phospholipids of *S. durissima*[a]

Compound[b]	ECL	Fatty acids	Per cent (by wt) in phospholipids	Distribution in phospholipid classes[c,d]			
				PI/PS	PC	PG	PE
1	14.00	Tetradecanoic (n-14:0; myristic)	0.9	–	–	2.0	2.1
2	14.60	13-Methyltetradecanoic (iso-15:0)	4.0	4.4	1.5	6.8	3.5
3	14.66	12-Methyltetradecanoic (anteiso-15:0)	1.9	1.9	–	3.5	1.9
4	15.00	Pentadecanoic (n-15:0)	0.8	2.4	–	0.8	–
5	15.59	14-Methylpentadecanoic (iso-16:0)	2.5	1.9	2.5	4.0	2.5
6	15.72	9-Hexadecanoic (Δ^9-16:1)	1.0	1.5	–	1.9	1.0
7	16.00	Hexadecanoic (n-16:0)	0.7	–	1.4	1.5	–
8	16.52	15-Methylhexadecanoic (iso-17:0)	3.3	2.5	3.5	3.7	3.2
9	16.57	14-Methylhexadecanoic (anteiso-17:0)	1.0	0.9	1.0	1.3	1.1
10	16.70	6-Heptadecanoic (Δ^6-17:1)	1.5	1.2	1.6	2.2	1.4
11	18.00	Octadecanoic (n-18:0, Stearic)	2.5	2.9	1.6	2.6	2.0
12	18.41	11-Methyloctadecanoic (11-Me-18:0)	6.7	6.7	3.0	11.2	6.1
13*	24.61	19-Methyltetracosanoic (19-Me-24:0)	0.5	0.5	–	0.7	0.9
14*	25.34	19-Methyl-5-pentacosenoic (19-Me-Δ^5-25:1)	5.0	5.2	10.1	2.4	2.7
15*	25.61	15-Methylpentacosanoic (19-Me-25:0)	4.7	4.5	9.6	2.2	2.5
16*	26.35	19-Methyl-5-hexacosenoic (19-Me-Δ^5-26:1)	19.3	24.7	30.6	9.9	11.3
17*	26.61	19-Methylhexacosanoic (19-Me-26:0)	7.5	5.3	4.1	7.7	13.0
18*	27.34	19-Methyl-5-heptacosenoic (19-Me-Δ^5-27:1)	7.8	5.5	8.2	7.1	12.1
19*	27.60	19-Methylheptacosanoic (19-Me-27:0)	12.3	10.4	10.8	11.8	12.9
20*	27.78	19,21-Dimethylheptacosanoic (19-Me,21-Me-27:0)	2.9	3.9	–	3.0	4.5
21*	28.60	20-Methyloctacosanoic (20-Me-28:0)	1.9	2.5	–	2.2	2.7
22*	28.79	20,22-Dimethyloctacosanoic (20-Me,22-Me-28:0)	1.9	1.8	3.1	0.9	2.0

[a]An asterisk after the compound number indicates that the acid is hitherto unknown.

[b]Identified minor (<0.5%) acids: 23) n-13:0 (ECL 13.00), 24) n-17:0 (ECL 17.00), 25) Δ^{10}-18:1 (ECL 17.72), 26) n-19:0 (ECL 19.00), 27) n-21:0 (ECL 21.00).

[c]Weight per cent (averages from 3 determinations).

[d]"–" implies that the acid occurred in a minor (less than 0.5%) amount.

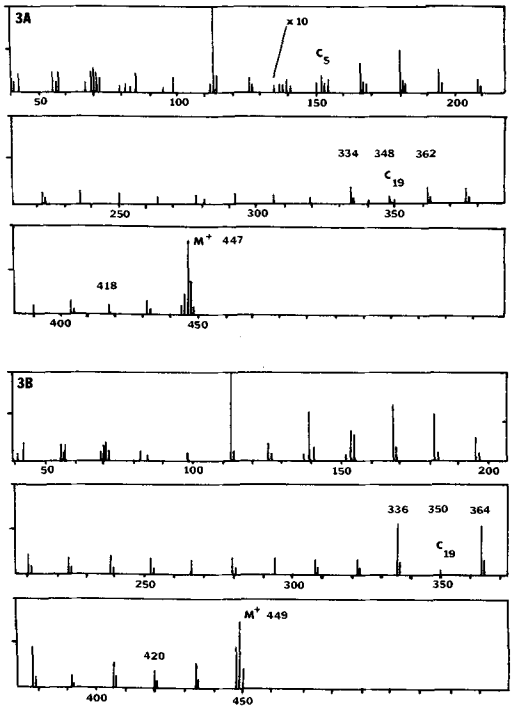


FIG. 3. (A) Mass spectrum of N-(19-methyl-5-pentacosenoyl)pyrrolidine (acid 14, Table 2). (B) Mass spectrum of N-(19-methyl-5-hexacosenoyl)pyrrolidine (acid 15, Table 2).

absolute configuration of C-19.

Compound 15 also was obtained by catalytic hydrogenation of the above-mentioned acid 14. The mass spectrum of pyrrolidide showed a molecular ion peak at m/z 449, typical for saturated C_{26} fatty acid pyrrolidides. The branching at C_{19} was confirmed by observing a peak of reduced intensity at C_{19} (m/z 350) surrounded by enhanced fragment peaks. All these results showed compound 15 to be 19-methylpentacosanoic acid.

The major demospongic acid found in the mixture was compound 16 (methyl ester M^+ 422). The mass spectrum of its pyrrolidide showed the molecular ion at m/z 461, characteristic for monounsaturated C_{27} acids. The presence of a double bond at C_5 was indicated again by a 12 amu difference between C_4 (m/z 140) and C_5 (m/z 152). Its IR spectrum gave no prominent absorption at 980-968 cm^{-1}, indicating a *cis* rather than a *trans* orientation. The position of branching was deduced first by observing a fragment peak of reduced intensity at C_{19} (m/z 348), accompanied by heightened flanking fragment peaks (Fig. 5A). Hydrogenation of the acid yielded compound 17 of the natural fatty acid mixture. In this case the

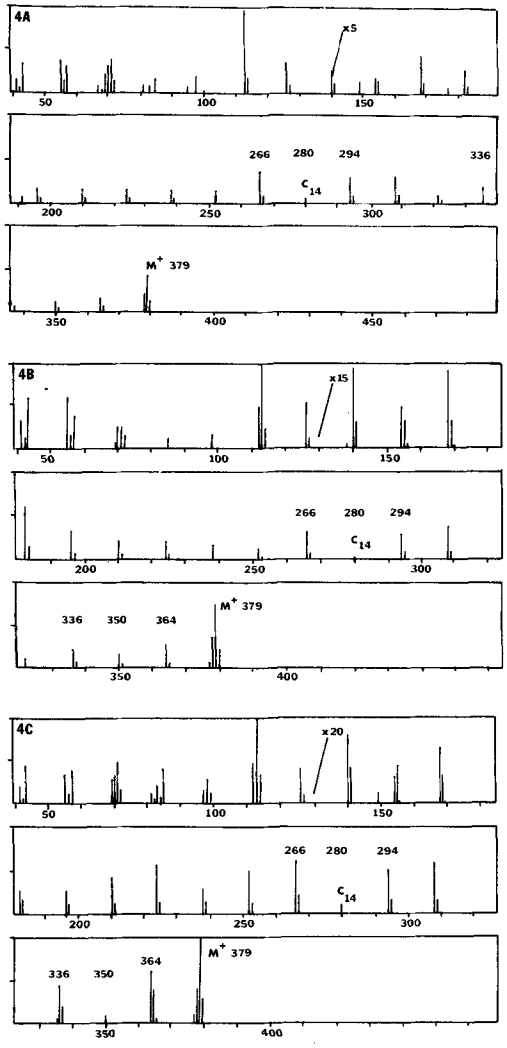
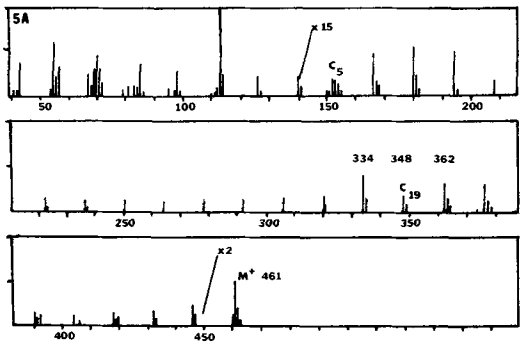


FIG. 4. (A) Mass spectrum of the pyrrolidide of the monofunctional degradation product from 19-methyl-5-pentacosenoic acid. (B) Mass spectrum of the pyrrolidide of 14-methyleicosenoic acid. (C) Mass spectrum of the pyrrolidide of 14,18-dimethylnonadecanoic acid.

fragment peak of diminished intensity at C_{19} shifted 2 amu to m/z 350 as expected (Fig. 5B).

In order to verify the position of the double bond, $NaIO_4/KMnO_4$ oxidative degradation of our acid was carried out which was purified by HPLC. The monofunctional degradation product was a C_{22} (methyl ester M^+ 354), in accordance with our assignment. The pyrrolidide mass spectrum of this product showed a molecular ion at m/z 393 and a methyl branching at C_{14} (m/z 280). Enhanced ions at C_{13} (m/z 266) and C_{15} (m/z 294) also were present (Fig. 6A). Since this is the major component of the mixture, we



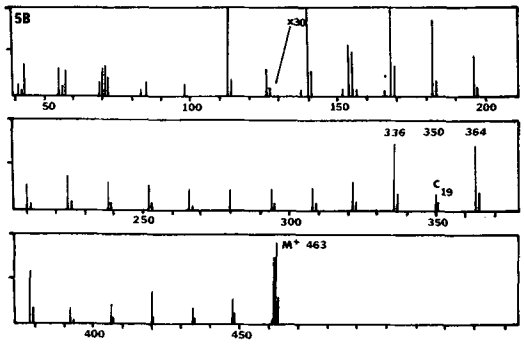
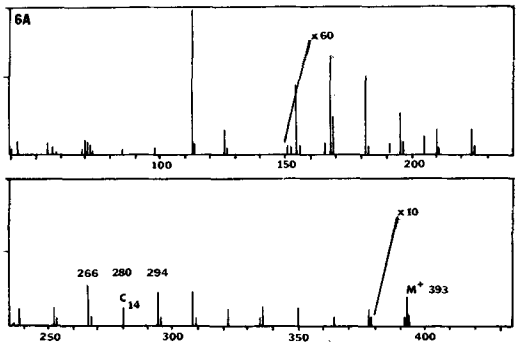


FIG. 5. (A) Mass spectrum of N-(19-methyl-5-hexacosenoyl)pyrrolidine. (B) Mass spectrum of N-(19-methylhexacosanoyl)pyrrolidine.



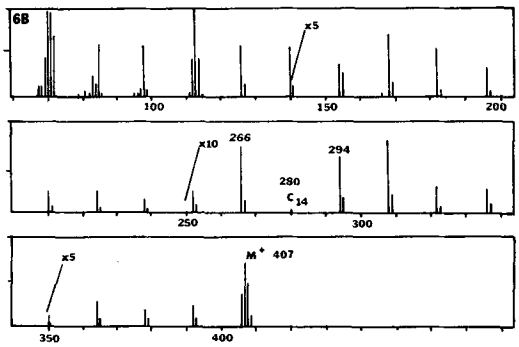


FIG. 6. (A) Mass spectrum of the pyrrolidide of the monofunctional degradation product from 19-methyl-5-hexacosenoic acid (acid 16, Table 2). (B) Mass spectrum of the pyrrolidide of the monofunctional degradation product from 19-methyl-5-heptacosenoic acid (acid 18, Table 2).

carried out the synthesis (Fig. 2) of the degradation product, methyl 14-methylheneicosanoate (see Exp. Section), in order to assign an unambiguous structure of the molecule. The synthetic methyl branched product was identical in all respects to the natural double bond degradation product, establishing the structure of the parent major acid 16 as *Z*-19-methyl-5-hexacosenoic acid. This result also indicates the structure of its saturated analog 17, present in the natural mixture, as 19-methylhexacosanoic acid.

The presence in the sponge of trace amounts of a similarly branched acid (acid 13, methyl ester M^+ 396, typical for a C_{25} acid) also was demonstrated. The molecular ion of its pyrrolidide derivative was found at m/z 435, verifying the occurrence of a C_{25} acid. A fragment peak with reduced intensity at m/z 350 indicated the position of branching at C_{19}. Fragment peaks with higher intensities also were observed at C_{18} (m/z 336) and C_{20} (m/z 364). Therefore, the assigned structure is 19-methyltetracosanoic acid (13).

Another very long chain phospholipid component (acid 18, methyl ester M^+ 436, hydrogenated methyl ester M^+ 438) in this series of 19-methyl branched analogs is a monounsaturated C_{28} acid. A double bond at C_5 was detected by observing a 12 amu difference between the most intense peaks of C_4 (m/z 140) and C_5 (m/z 152) as anticipated in the pyrrolidide mass spectrum of the acid. A diminished fragment peak also was seen at C_{19} (m/z 348). On hydrogenation, the fragment peak with reduced intensity was shifted to m/z 350. Enhanced peaks at C_{18} and C_{20} were shifted by 2 amu. The IR spectrum of the purified compound by HPLC again indicated a *cis* double bond. The oxidative degradation of the parent compound produced a C_{23} acid (methyl ester M^+ 368) as the monofunctional reaction product. Its pyrrolidide (M^+ 407) showed the expected diminished peak at C_{14} (m/z 280) (Fig. 6B). These results lead to the conclusion that compound 18 was indeed a branched Δ^5 phospholipid acid, namely 19-methyl-5-heptacosenoic acid. Compound 19 also was obtained by catalytic hydrogenation of acid 18. From the above-mentioned observations its structure was assigned as *Z*-19-methyl-

heptacosanoic acid.

A second long chain, monobranched fatty acid which did not change upon hydrogenation (acid 21, methyl ester M^+ 452) also was detected in the mixture. The pyrrolidide spectrum exhibited a molecular ion peak at m/z 491, featuring the presence of a C_{29} acid and a fragment peak with reduced intensity at m/z 364, this time pointing to a methyl branch at C_{20} rather than C_{19} (Fig. 7). Flanking fragment peaks with increased intensities were observed at C_{19} (m/z 350) and C_{21} (m/z 378). Therefore the structure of this new component is established as 20-methyloctacosanoic acid (21).

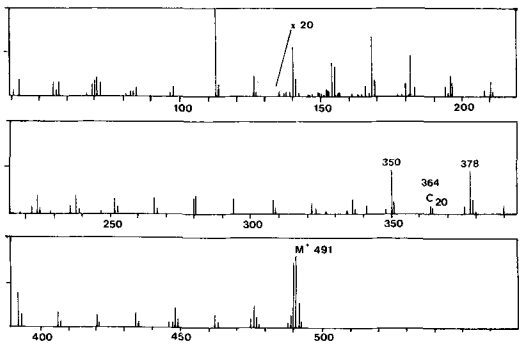


FIG. 7. Mass spectrum of N-(20-methyloctacosanoyl) pyrrolidine (acid 21, Table 2).

Finally we also encountered small amounts of 2 branched C_{29} and C_{30} saturated demospongic acids with unusually small ECL values (20 and 22 in Table 2). The pyrrolidide spectrum of 20 (methyl ester M^+ 452) showed a molecular ion peak m/z 491 (Fig. 8A), characteristic for a C_{29} acid derivative. The fragment peaks with reduced intensities at m/z 350 and m/z 392 indicate 2 methyl branchings rather than one, at C-19 and C-21 (22) in accordance with the ECL value (Fig. 8A). Enhanced fragment peaks also were observed at m/z 336, m/z 364, m/z 378 and at m/z 406. The pyrrolidide mass spectrum of the second compound with an unusually short ECL value (acid 22, methyl ester M^+ 466) showed a molecular ion at m/z 505 and indicated a saturated C_{30} acid. The fragment peaks with lowered intensities at m/z 364 and m/z 406 again pointed to two branchings, this time at C-20 and C-22 (Fig. 8B). Elevated fragment peaks also were observed at m/z 350, m/z 378, m/z 392 and m/z 420. Given the relatively short ECL values (23) and mass spectral properties (24), the structures of these 2 novel phospholipid components 20 and 22 were assigned tentatively as 19,21-dimethylheptacosanoic acid and 20,22-dimethyloctacosanoic acid respectively. To our knowledge, fatty acids with such a dimethylation pattern are not known in nature.

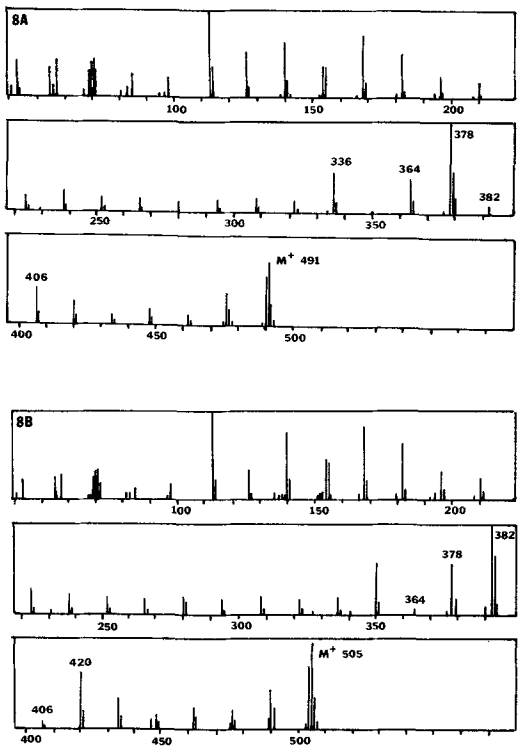


FIG. 8. (A) Mass spectrum of N-(19,21-dimethylheptacosenoyl)pyrrolidine (acid 20, Table 2). (B) Mass spectrum of N-(20,22-dimethyloctacosanoyl)pyrrolidine (acid 22, Table 2).

Major phospholipids present in the sponge (Table 1) are PE, PS and PC. Our present and previous studies demonstrate that high percentages of PE and PS seem to be characteristic features of sponges. PG and PI usually are found in relatively small amounts and are known to occur in bacterial membranes. Therefore, the presence of significant quantities of PG and PI in our extract implies the presence of bacteria, probably in symbiosis with our sponge. The detection of typical bacterial components, iso and anteiso acids in the total phospholipid acid mixture, also suggests such a coexistence. These acids in our phospholipid fraction (iso-15:0, anteiso-15:0, iso-16:0, iso-17:0, and anteiso-17:0) contribute 12.1% of the total mixture. 11-Methyloctadecanoic acid which comprises 6.5% of the total mixture also may come from bacteria (9). In fact, relatively large quantities of these acids are in the PG fraction (Table 2) which further supports a

bacterial origin. Based on the analytical studies carried out so far, marine bacteria do not possess very long chain fatty acids in their lipids. On the other hand, our studies (9,10,13) show that typical sponge phospholipids (PE, PS, and sometimes PC) always contain relatively smaller amounts of normal chain and larger amounts of very long chain demospongic acids. These findings lead us to believe that the novel C_{24-30} fatty acids are from sponge phospholipids and not from lipids extracted from the bacterial symbionts.

This is the first time that large quantities (30.7% of the total fatty acid mixture) of very long chain Δ^5 acids rather than $\Delta^{5,9}$ of diunsaturated acids have been found in sponges. According to the studies by Litchfield and his co-workers on demospongic acids, a double bond at C_9 is first introduced, followed by a second double bond at C_5 (26). Our new group of Δ^5 monounsaturated demospongic acids opens up the possibility of a different biosynthetic pathway in *Strongylophora durissima*. So far, we have encountered different novel mono- and diunsaturated straight chain, branched, methoxy, cyclopropane (9-13) or brominated (27) phospholipid fatty acids with one common feature: a very long hydrocarbon chain up to 30 carbons which seems to be unique to these primitive marine organisms.

A recent article (28) on the evolution of the phospholipid composition of marine invertebrates points out that the ascent up the evolutionary scale is associated with changes in the composition of phospholipids, and an increase of the total PC and PE together with a decrease of PS (in sponges and arthropods in comparison with tunicates, respectively) is observed. It also is stated that an increase in the degree of saturation is accompanied by the disappearance of demospongic acids. The presumed causes of these alterations are changes of temperature, oxygen concentration and degree of differentiation of tissues during the course of evolution. Further investigations on morphological and biosynthetic implications of our findings are under way.

ACKNOWLEDGMENTS

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REFERENCES

1. Djerassi, C. (1981) Pure Appl. Chem. 53, 873-890.
2. Withers, N.W. (1983) in Marine Natural Products (Scheuer, P.J., ed.) Vol. V, pp. 88-127, Academic Press, New York.
3. Bergmann, W., and Swift, A.N. (1951) J. Org. Chem. 16, 1206-1221.
4. Litchfield C. (1976) in Aspects of Marine Biology (Harrison, R.W., and Cowden, R.R., eds.) p. 183, Academic Press, New York.
5. Morales, R.W., and Litchfield, C. (1976) Biochim. Biophys. Acta 431, 206-216.
6. Litchfield, C., and Marcantonio, E.E. (1978) Lipids 13, 199-202.
7. Litchfield, C.; Tyszkiewitz, J.; Marcantonio, E.E., and Noto, G. (1979) Lipids 14, 619-622.
8. Bergquist, P.R. (1984) Biochem. Sys. Ecology, in press.
9. Walkup, R.D.; Jamieson, G.C.; Ratcliff, M.R., and Djerassi, C. (1981) Lipids 16, 631-646.
10. Ayanoglu, E.; Walkup, R.D.; Sica, D., and Djerassi, C. (1982) Lipids 17, 617-625.
11. Lankelma, J.; Ayanoglu, E., and Djerassi, C. (1983) Lipids 18, 853-858.
12. Ayanoglu, E.; Kornsprobst, J.M.; Aboud-Bichara, A., and Djerassi, C. (1983) Tet. Letters 24, 1111-1114.
13. Ayanoglu, E.; Popov, S.; Kornprobst, J.M.; Aboud-Bichara, A., and Djerassi, C. (1983) Lipids 18, 830-836.
14. Bartolotto, M.; Braekman, J.S.; Daloze, D., and Tursch, B. (1978) Bull. Soc. Chim. Belg. 87, 539.
15. Theobald, N., and Djerassi, C. (1978) Tetrahedron Lett. 4369-4372.
16. Li, L.N., and Djerassi, C. (1981) Tetrahedron Lett. 4639-4642.
17. Privett, O.S.; Dougherty, K.A.; Erdahl, W.L., and Stolyhwo, A. (1973) J. Am. Chem. Soc. 50, 516-520.
18. Barlett, G.R. (1959) J. Biol. Chem. 234, 466-468.
19. Skipski, V.P.; Peterson, R.F., and Barclay, M. (1964) Biochem. J. 90, 374-378.
20. Carreau, J.P., and Dubacq, J.P. (1978) J. Chromatog. 151, 384-390.
21. Morrison, W.R., and Smith, L.M. (1964) J. Lipid Res. 5, 600-608.
22. Albro, P.W., and Dittmer, J.C. (1969) Biochemistry 8, 394-404.
23. Ackman, R.G. (1972) Prog. Chem. Fats Other Lipids 12, 167-284.
24. Andersson, B.A. (1978) Prog. Chem. Fats Other Lipids 16, 279-308.
25. Dyer, J.R. (1965) in Applications of Absorption Spectroscopy of Organic Compounds, pp. 30-31, Prentice-Hall, Englewood Cliffs, NJ.
26. Morales, R.W., and Litchfield, C. (1977) Lipids 12, 570-576.
27. Wijekoon, D.; Ayanoglu, E. and Djerassi, C. (1981) Tet. Letters 25, 3285-3287.
28. Kostetski, E.Y. and Shchipunov, Y.A. (1983) Zh. Evol. Biokhim. Fiziol. 19, 8-15.

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Effect of High Doses of Synthetic Estrogen On Lipid Metabolism in Castrated Male Rats

M. NISHIKAWA,* K. SEKI, Y. MATSUZAWA, Y. MINAMI, S. KAWATA, S. MIYOSHI, Y. IMAI, R. SAITOH, S. NODA, S. TAMURA and S. TARUI, *The Second Department of Internal Medicine, Osaka University Medical School, Fukushima, Fukushima-ku, Osaka, Japan, 553*

ABSTRACT

The mechanism by which high doses of estrogen influences lipid metabolism was studied with a microtubular blocking agent. Castrated male rats received oral injection daily for 14 days of 3 mg hexestrol in olive oil, or oil alone as controls. About half of the animals in each group were injected intraperitoneally with 4 mg/100 g body weight colchicine 3 hr before they were killed. Hexestrol treatment caused an accumulation of esterified cholesterol in the liver while it decreased those in serum. Triglyceride concentrations slightly decreased in the liver but were unaffected in serum. On polyacrylamide-gel disc electrophoresis, the peaks of high density lipoproteins (HDL) and low density lipoproteins (LDL) were decreased remarkably. Electron microscopic examination of hepatocytes revealed electron-lucent lipid droplets in the cytoplasm.

After a colchicine treatment of the control animals, concentrations of esterified cholesterol and triglycerides markedly increased in the liver, while those in serum decreased. Electron microscopic examination of hepatocytes revealed numerous secretory vesicles filled with nascent VLDL. In hexestrol-treated animals, the colchicine treatment was associated with marked decreases in serum-esterified cholesterol and triglyceride as seen in the controls. However, there were no further increases of esterified cholesterol in the liver, and the increase of triglycerides was slight. Electron microscopic examination showed less secretory droplets than in the controls.

These data suggest that very low density lipoproteins (VLDL) synthesis in the liver of hexestrol treated rats was inhibited. An accumulation of esterified cholesterol with a marked decrease in serum could not be accounted for by the inhibition of lipoproteins secretion, but rather by their enhanced entry into the liver.

Lipids 19:777-783, 1984.

INTRODUCTION

Many studies have shown that estrogen influences lipid metabolism (1-4), and administration of higher doses of estrogen causes hypolipidemia, especially hypocholesterolemia (5-9). Recently, we reported that the treatment of prostatic cancer with high doses of synthetic estrogen caused nonalcoholic steatohepatitis, in which marked accumulation of lipid droplets and steatonecrosis were seen in the liver (10). Since most of the patients in our study showed hypocholesterolemia, the occurrence of steatohepatitis and changes in the level of serum lipids should depend on the alteration of lipoprotein metabolism by the effect of estrogen. Lipoproteins secreted from the liver and the small intestine to the blood stream are degraded by lipoprotein lipase and hepatic-triglyceride lipase, and parts of them are taken up by hepatocytes through lipoprotein receptors (11). Therefore, investigation of these steps may throw further light on the altered-lipid metabolism in the treatment of estrogen.

In order to discover the mechanism for the creation of steatohepatitis and hypocholesterolemia by estrogen, we treated rats with high doses of estrogen and investigated both serum and liver lipids in combination with morphological studies. In addition, the effect of colchicine, which is known to inhibit the final steps of lipoprotein secretion (12,13), was investigated to elucidate which steps of lipoprotein metabolism are mainly involved in the changes of serum and liver lipids.

*To whom correspondence should be addressed.

MATERIALS AND METHODS

Treatment of Animals

Male Wistar rats weighing 120-130 g (Nihon Dobutsu Co. Ltd.) were castrated under thiamylal anesthesia (5 mg/100 g body weight, intraperitoneal injection) and were housed in wire mesh cages illuminated from 7 a.m. to 9 p.m. All animals had free access to water and standard rat chow. After 4-day intervals, the animals received daily oral injections of 3 mg hexestrol (Wako Pure Chemical Ind. Co. Ltd.) dissolved in olive oil (10 mg/ml), or oil alone as controls for 14 days. On the 15th day, following overnight fasting, the animals were killed by exanguination from the heart. Three hours before they were killed, about half of the

animals in each group were injected intraperitoneally with 4 mg/100 g body weight colchicine (10 mg/ml, dissolved in 0.9% NaCl immediately before use, Wako Pure Chemical Ind. Co. Ltd.).

Hepatic Morphology

Immediately after the collection of blood, laparotomy was performed and the liver removed. The liver from each animal was divided into 3 pieces for cytochemical studies (pooled at −20 C), for light microscopic study (fixed in 10% formalin) and for electron microscopic study. Livers for electron microscopic study were fixed in 2% osmium oxide or 1% osmium oxide after fixation with 2.5% glutal aldehyde, embedded in Epon, and stained with uranyl acetate and lead citrate.

Serum Lipid and Lipoprotein Analysis

The blood from each animal was collected, allowed to clot at room temperature and centrifuged at 1500 g for 15 min. The serum thus obtained from each animal was pooled at 4 C. Serum lipid analysis was determined enzymatically, and HDL cholesterol was determined enzymatically after the precipitation of VLDL and LDL by heparin-Ca^{++}. Analysis of serum lipoproteins was performed by polyacrylamide-gel disc electrophoresis according to the method of Narayan (14).

Liver Lipid Analysis

Total lipids in the animal liver (400-500 mg fresh weight) were extracted by the method of Folch (15). The amount of total lipids was estimated gravimetrically with an electromicrobalance (Chan) after drying an aliquot of the sample at 130 C for 3 min. The amount of cholesterol was determined by the Zurkowski method (16). Esterified cholesterol and triglyceride analyses were performed by the ester-linkage determination after separation by thin layer chromatography (TLC) (17). The amount of phospholipids was determined by the method of Chen (18).

Liver Protein Analysis

The amount of liver protein was determined by the method of Lowry (19) after the homogenization of the liver (100-150 mg) in a 0.25 M sucrose buffer.

Statistical Analysis

Statistical differences were analyzed using Student's t-test.

TABLE 1

Effects of Hexestrol and Colchicine on Concentrations of Serum Lipids

Animal group	Treatment	No. of animals	Total cholesterol (mg/dl)	Free cholesterol (mg/dl)	Esterified cholesterol (mg/dl)	Phospholipid (mg/dl)	Triglyceride (mg/dl)	HDL cholesterol (mg/dl)
I		8	81 ± 5	18 ± 1	63 ± 4	119 ± 9	54 ± 9	67 ± 4
II	Colchicine	6	63 ± 4	18 ± 1	45 ± 3	114 ± 5	20 ± 3	61 ± 4
III	Hexestrol	11	41 ± 5	20 ± 3	21 ± 3	85 ± 18	69 ± 13	15 ± 2
IV	Hexestrol + colchicine	7	13 ± 1	13 ± 2	N.D.	67 ± 4	16 ± 3	21 ± 1
Statistical analysis								
I vs III			$P < 0.001$	N.S.	$P < 0.001$	N.S.	N.S.	$P < 0.001$
I vs II			$P < 0.02$	N.S.	$P < 0.005$	N.S.	$P < 0.005$	N.S.
III vs IV			$P < 0.001$	N.S.	$P < 0.001$	N.S.	$P < 0.005$	N.S.

All animals were treated with olive oil. Values are mean ±S.E.M. N.D. = not detectable. N.S. = not significant.

RESULTS

Changes in Serum Lipids and Lipoproteins

Table 1 shows the effects of 3 mg hexestrol administration with or without colchicine. Three mg of hexestrol caused a marked decrease in serum-cholesterol concentrations from 81 to 41 mg/dl. The decrease of serum cholesterol was accounted for by the decrease of esterified cholesterol from 63 to 21 mg/dl. Also, the level of HDL cholesterol was remarkably reduced from 67 to 15 mg/dl. On the other hand, the level of serum triglyceride was increased slightly but not statistically. Patterns of lipoproteins on polyacrylamide-gel disc electrophoresis are shown in Figure 1. Peaks of HDL and LDL were reduced by treatment with hexestrol. After the colchicine treatment, marked decreases of serum-total cholesterol, esterified cholesterol and triglyceride were observed both in the hexestrol-treated animals and in the controls.

Changes in Liver Lipids

Table 2 shows the effects of hexestrol with or without colchicine in the liver. Administration of 3 mg hexestrol resulted in a marked increase in liver-esterified cholesterol concentrations from 26 to 115 μmol/10 g liver. Concentrations of triglyceride decreased slightly, from 59 to 33 μmol/10 g liver. After the colchicine treatment, significant increases of total lipids (from 38 to 55 μg/mg liver), phospholipids (24 to 36 μg/mg liver), free cholesterol (22 to

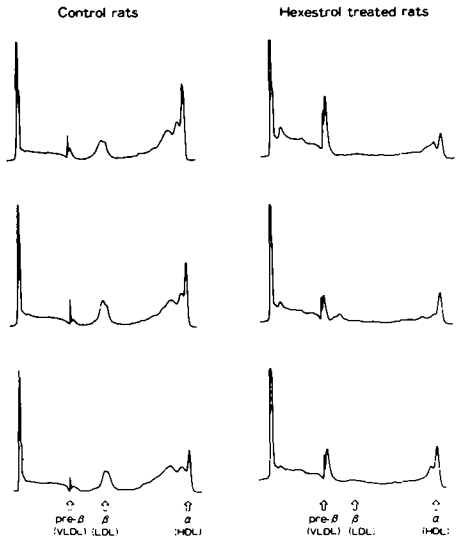

FIG. 1. Polyacrylamide-gel disc electrophoretic pattern of lipoproteins in hexestrol-treated rats. The peaks of HDL and LDL are lower than in the controls.

TABLE 2

Effect of Hexestrol and Colchicine on Concentrations of Liver Protein and Lipids

Animal group	Treatment	No. of animals	Protein (μg/mg)	Total lipid (μg/mg)	Phospholipid (μg/mg)	Total cholesterol (μmol/10 g liver)	Free cholesterol (μmol/10 g liver)	Esterified cholesterol (μmol/10 g liver)	Triglyceride (μmol/10 g liver)
I		8	173 ± 6	38 ± 1	24 ± 1	49 ± 2	22 ± 4	26 ± 2	59 ± 9
II	Colchicine	6	186 ± 5	55 ± 1	36 ± 1	107 ± 13	70 ± 8	36 ± 4	111 ± 10
III	Hexestrol	11	169 ± 8	38 ± 2	25 ± 1	144 ± 17	29 ± 6	115 ± 15	33 ± 3
IV	Hexestrol + Colchicine	7	166 ± 5	48 ± 3	32 ± 1	149 ± 11	55 ± 3	93 ± 10	42 ± 1
Statistical analysis									
I vs III			N.S.	N.S.	N.S.	$P < 0.001$	N.S.	$P < 0.001$	$P < 0.02$
I vs II			N.S.	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.05$	$P < 0.005$
III vs IV			N.S.	$P < 0.05$	$P < 0.001$	N.S.	$P < 0.005$	N.S.	$P < 0.02$

All animals were treated with olive oil. Values are mean ±S.E.M. N.S. = not significant.

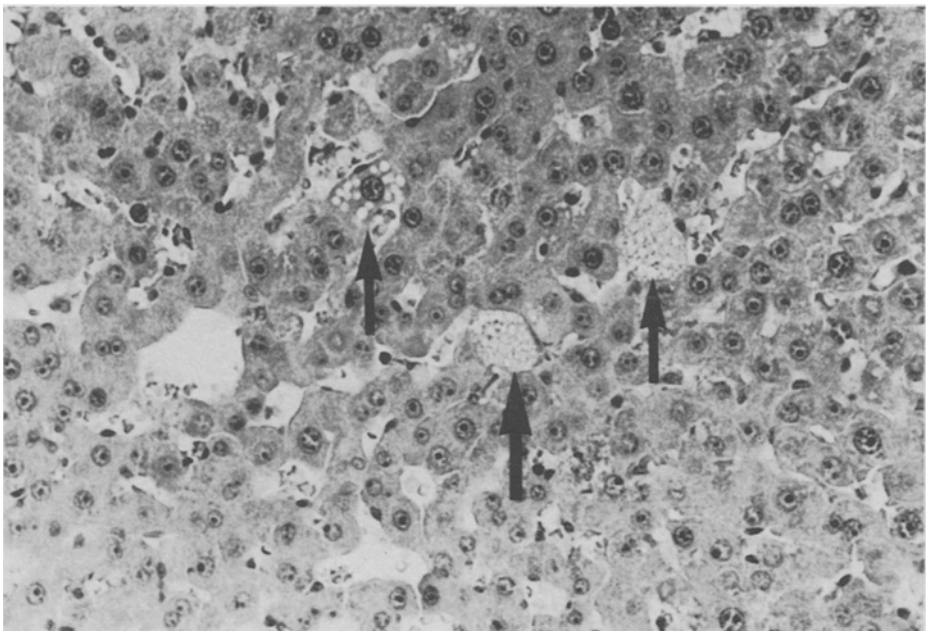

FIG. 2. Liver of hexestrol-treated rat, showing hepatocytes containing numerous lipid droplets (arrows). There are not inflammatory or degenerated signs in the other hepatocytes. (HE stain × 100)

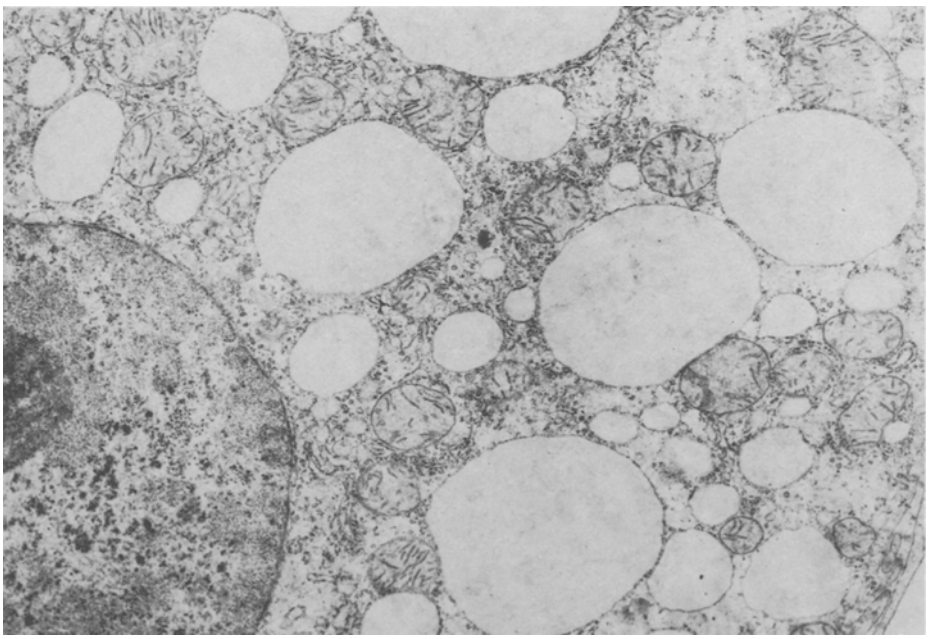

FIG. 3. Electron micrograph of the hepatocyte containing lipid droplets, showing electron-lucent lipid droplets with a single membrane. (×7000)

70 μmol/10 g liver), esterified cholesterol (26 to 36 μmol/10 g liver) and triglyceride (59 to 111 μmol/10 g liver) were observed in the control animals. In hexestrol-treated animals, colchicine produced the same changes in phospholipids (from 25 to 32 μg/mg liver) and free cholesterol (29 to 55 μmol/10 g liver) as was the case in the control animals. However, esterified-cholesterol concentrations were not increased (from 115 to 93 μmol/10 g liver) and the increase in triglyceride was slight (33 to 42 μmol/10 g liver).

Changes in Morphology of the Liver

Light microscopic examination of hexestrol-treated animals revealed a few hepatocytes containing lipid droplets (Fig. 2), while under the electron microscope, numerous electron-lucent lipid droplets with a single membrane were found in the cytoplasm (Fig. 3). After the colchicine treatment, electron microscopic examination of the hepatocytes of control animals revealed Golgi elements with a higher load of nascent-VLDL particles and a large population of VLDL-containing secretion vacuoles. These were found scattered throughout the cytoplasm (Fig. 4). In hexestrol treated animals, the electron microscopic examination of the liver revealed, however, only a few VLDL vesicles in the cytoplasm in comparison with controls (Fig. 5).

DISCUSSION

Treatment of rats with high doses of hexestrol caused a marked reduction of esterified-cholesterol concentrations in serum, which was accounted for by decreases in HDL and LDL in confirmation of previous reports (5,8,9). In addition to the changes of lipids and lipoproteins in serum, esterified-cholesterol concentrations markedly increased in the liver while the concentrations of triglycerides decreased slightly. Light and electron microscopic examinations of the liver revealed hepatocytes containing substantial numbers of lipid droplets.

Several mechanisms should be involved in the occurrence of those changes following the administration of high doses of estrogen. Although previous reports have suggested stimulated catabolism or enhanced removal of lipoproteins might be the cause of hypolipidemia (5,8,9), the mechanism of the hypolipidemic effect of high doses of estrogen has not been clarified completely. In addition, the accumulation of esterified cholesterol in the liver could not be explained by stimulated catabolism.

Esterified cholesterol and triglyceride of rats are secreted mainly in $d < 1.01$ lipoproteins, and their secretion is completely inhibited by colchicine (20). Colchicine treatment caused marked decreases of serum-esterified cholesterol and triglyceride in hexestrol-treated animals, similar to the results in the controls. In the

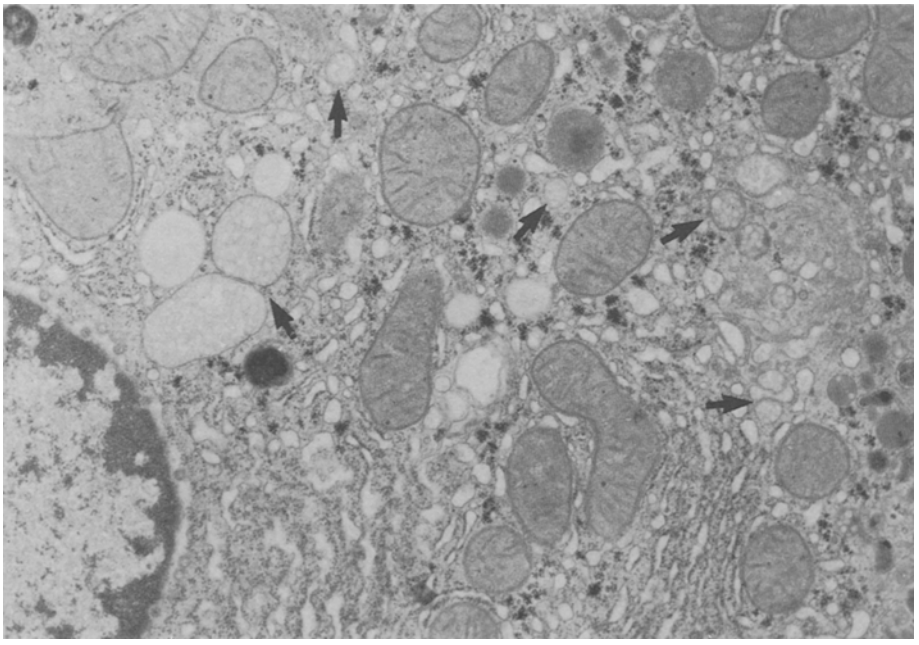

FIG. 4. Electron micrograph of the hepatocyte of control animal after a colchicine treatment. Numerous secretory vesicles containing nascent VLDL are shown. (×7000)

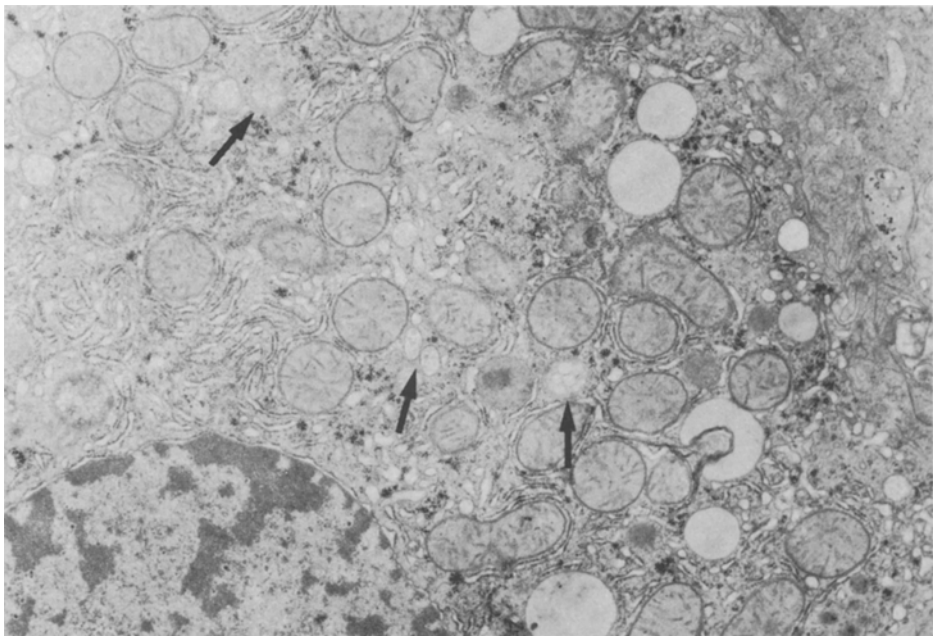

FIG. 5. Electron micrograph of the hepatocyte of hexestrol-treated rat after a colchicine treatment. There are a few VLDL vesicles as compared with those in the controls. (×7000)

liver, triglyceride concentrations increased slightly, while esterified-cholesterol concentrations did not increase further due to the administration of colchicine in hexestrol-treated animals. Electron microscopic examination revealed less VLDL particles in the hepatocytes of hexestrol-treated animals than in those of controls. This suggests that hexestrol does inhibit VLDL synthesis. Therefore, the marked increase in the liver and decrease in the serum of esterified cholesterol accompanied by the decrease of HDL and LDL in serum could be explained only by the enhanced entry of these lipoproteins to the liver from circulation. This is supported by the observation of Wilcox (9), who reported enhanced removal of Apo-E rich subfraction of HDL from circulation accompanied by large doses of ethynyl estradiol.

Other possible factors affecting the mechanism of lipid alteration might be considered, e.g. toxic effect of estrogen or the cholestatic effect of estrogen. Food consumption of rats treated with hexestrol decreased slightly. However, there were not degenerated nor inflammatory signs in the liver of hexestrol-treated rats except for hepatocytes containing lipid droplets (Fig. 2). Consequently, 3 mg of hexestrol cannot be considered as having substantial toxic effect on the liver.

There are several reports concerning the cholestatic effect of estrogen (21). However, in this experiment there were no signs of cholestasis in the liver treated with this drug.

The fate of esterified cholesterol is hydrolyzed by cholesterol esterase in lysosome (22). In our previous reports, 4,4′-diethylaminoethoxyhexestrol, which also was a compound of hexestrol, caused marked accumulations of phospholipids and esterified cholesterol in the liver accompanied by peculiar morphological changes showing a large number of multilamellar inclusion bodies in the hepatocytes (23,24). The mechanism of this accumulation was clarified to be the inhibition of lysosomal lipid hydrolytic enzyme (25). From another point of view, accumulation of esterified cholesterol in the liver might be accounted for by the inhibition of cholesterol esterase.

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REFERENCES

1. Rössner, S.; Larsson-Cohn, U.; Carlson, L.A., and Boberg, J. (1971) Acta Med. Scand. 190, 301-305.
2. Kekki, M., and Nikkilä, E.A. (1971) Metabolism 20, 878-889.
3. Hamosh, M., and Hamosh, P. (1975) J. Clin. Invest. 55, 1132-1135.
4. Wallentin, L., and Larsson-Cohn, U. (1977) Acta

Endocr. 86, 597-607.
5. Fewster, M.E.; Pirrie, R.E., and Turner, D.A. (1967) Endocrinology 80, 263-271.
6. Ferreri, L.F., and Naito, H.K. (1978) Endocrinology 102, 1621-1627.
7. Davis, R.A., and Roheim, P.S. (1978) Atherosclerosis 30, 293-299.
8. Chao, Y.; Windler, E.E.; Chen, G.C., and Havel, R.J. (1979) J. Biol. Chem. 154, 11360-11366.
9. Wilcox, H.G.; Kenagy, R.; Weinstein, I., and Heimberg, M. (1981) Biochem. Biophys. Acta 666, 348-355.
10. Seki, K.; Minami, Y.; Nishikawa, M.; Kawata, S.; Miyoshi, S.; Imai, Y., and Tarui, S. (1983) Gastroenterol. Japon. 18, 197-203.
11. Scow, R.O.; Blanchette-Mackie, E.J., and Smith, L.C. (1976) Circ. Res. 39, 149-162.
12. Stein, O., and Stein, Y. (1973) Biochem. Biophys. Acta 306, 142-147.
13. Redman, C.M.; Banergee, D.; Howell, K., and Palade, G.E. (1975) J. Cell Biol. 66, 42-59.
14. Narayan, K.A.; Creinin, H.L., and Kummerow, F.A. (1966) J. Lipid Res. 7, 150-157.
15. Folch, J.; Lee, M., and Stanley, G.H.S. (1957) J. Biol. Chem. 226, 497-509.
16. Zurkowski, P. (1964) Clin. Chem. 10, 451-453.
17. Rapport, M.M., and Alonzo, N. (1955) J. Biol. Chem. 217, 193-198.
18. Chen, P.S.; Toribara, T.Y., and Warner, H. (1956) Analy. Chem. 28, 1756-1758.
19. Lowry, O.H.; Rosebrough, N.J.; Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
20. Kampen, H.J.M. (1980) J. Lipid Res. 21, 671-680.
21. Sherlock, S. (1981) Disease of the Liver and Biliary System, 6th edn., pp. 312-317, Blackwell Scientific Publications, London.
22. Myant, N.B. (1981) The Biology of Cholesterol *and Related Steroids, 1st edn., pp.* 275, William Heinemann Medical Books, London.
23. Adachi, S.; Matsuzawa, Y.; Yokomura, T.; Ishikawa, K.; Uhara, S.; Yamamoto, A., and Nishikawa, M. (1972) Lipids 7, 1-7.
24. Matsuzawa, Y., and Hostetler, K.Y. (1980) J. Lipid Res. 21, 202-214.
25. Matsuzawa, Y., and Hostetler, K.Y. (1980) J. Biol. Chem. 255, 5190-5144.

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Reduction of Polyunsaturated Fatty Acid Hydroperoxides by Human Brain Glutathione Peroxidase

E. KAPLAN[*a] and **K. ANSARI**,[b] *Neuro-Immunology Research Laboratory and Neurology Service, Veterans' Administration Medical Center, Minneapolis, MN, U.S.A.; and Department of Neurology*[b] *and Department of Medicine*,[a] *University of Minnesota Health Sciences Center, Minneapolis, MN, U.S.A.*

ABSTRACT

Glutathione peroxidase (GSHPx) activity in the normal human brain was investigated using lipid hydroperoxides as substrates. Samples were obtained from autopsied frontal gray matter of 5 normal human males with no known central nervous system (CNS) disease. Aliquots were homogenized in 0.9% NaCl-0.5% Triton X-100, and the supernatant solution, obtained after centrifugation at 105,000 × g, was used for GSHPx assay. Glutathione peroxidase was measured by following the oxidation of NADPH at 340 nm. Hydroperoxides of linoleic, linolenic, gamma linolenic, 11,14 eicosodienoic, homo gamma linolenic, arachidonic, docosotetraenoic and docosohexaenoic acids were prepared and used as substrates. All these hydroperoxides were reduced by the brain GSHPx system, but at different rates. Gamma linolenic and docosotetraenoic hydroperoxides were reduced rapidly, whereas the peroxides of docosohexaenoic and 11,14 eicosodienoic were reduced at the lowest rate. Arachidonic hydroperoxide had the highest affinity for the enzyme and linolenic the lowest. Our results suggest that the brain GSHPx system is capable of reducing hydroperoxides of polyunsaturated fatty acids.
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INTRODUCTION

The brain contains a high concentration of polyunsaturated fatty acids (PUFA). These PUFA are important constituents of phospholipids of cell membranes (1,2). Analysis of brain phospholipids has shown that they are rich in the following PUFA: arachidonic (20:4ω6), docosotetraenoic (22:4ω3) and docosohexaenoic (22:6ω6) (1,2). PUFA are susceptible to toxic oxygen products (3); increased formation of hydroperoxides has been implicated in the development of senescence (3-6) and cellular damage (3,7-9). Removal of these harmful hydroperoxides from tissues may occur through the action of the enzyme glutathione peroxidase (GSHPx, EC 1.11.1.9) (3,10,11). Although the relevance of GSHPx in prevention of tissue lipid peroxidation has been questioned (12-14), many investigators (3,10,11) feel that this peroxidase is of importance in reduction of lipid hydroperoxides. It has been reported that GSHPx from liver and erythrocytes can reduce lipid peroxides (3,10,15,16). No studies have been performed on the ability of GSHPx in brain to reduce these fatty acid hydroperoxides. Since the brain does have a measurable quantity of GSHPx (17-19), we investigated the capacity of the enzyme in frontal gray matter of the human brain to reduce these hydroperoxides which may be produced in vivo non-enzymatically and/or enzymatically from their own PUFA. We have evaluated the reduction of 8 PUFA hydroperoxides by GSHPx present in normal human brain supernatant solution and compared these results with those obtained using H_2O_2 and t-butyl hydroperoxide as substrates.

*To whom correspondence should be addressed at Research Service, V.A. Medical Center, Minneapolis, MN 55417.

MATERIALS AND METHODS

Frontal gray matter samples from 5 autopsied normal human brains were used for this study. Individuals from whom samples were obtained ranged between 52 and 62 yrs of age, and all were males. Two of the 5 brains were analyzed for GSHPx within a few days of autopsy, and the other 3 samples had been stored at −80 C for 7 to 10 yr. We have found that GSHPx activity in normal frontal gray matter samples (excised during surgical approach for various intracranial lesions) and fresh autopsy human frontal aliquots were similar to those of frontal tissues which had been stored for 7 to 10 years (unpublished observations).

The chemicals Hepes, EDTA, NADPH, sodium azide, soybean lipoxygenase, glutathione reductase and t-butyl hydroperoxide were obtained from Sigma Chemical Co., St. Louis, Missouri. H_2O_2 was purchased from Fisher Scientific Prod., Pittsburgh, Pennsylvania. GSH and Triton X-100 were bought from Cal-Biochem., LaJolla, California. Hemoglobin standards and Drapkins reagent were obtained

from Hycel, Inc., Houston, Texas. The following PUFA were purchased from Nu-Chek-Prep Inc., Elysian, Minnesota: linoleic (18:2ω6), γ linolenic (18:3ω6), linolenic (18:3ω3), homo γ linolenic (20:3ω6), 11,14 eicosodienoic (20:2ω6), arachidonic (20:4ω6), docosotetraenoic (22:4ω6) and docosohexaenoic (22:6ω3). These PUFA were greater than 99% pure according to GC and TLC analysis performed by Nu-Chek-Prep Inc.

Enzyme assay. Tissue was homogenized in 9 vol of 0.9% NaCl-0.5% Triton X-100 using a Polytron (Brinkman, Westbury, New York) intermittently for a total time of one minute at the maximum speed setting. This homogenate was centrifuged at 105,000 × g for 90 min in a Beckman preparative ultracentrifuge. A 60 μl aliquot of the supernatant solution was used for GSHPx assay. GSHPx activity was determined by the method of Beutler (20) as modified by Prohaska and Ganther (17). Hepes-EDTA, pH 7.5, was substituted for phosphate buffer, pH 7.0 (Prohaska, J., personal communication). Final concentration of the components in a volume of 2.0 ml was: 0.1 M Hepes, 3.0 mM EDTA, 1.0 mM GSH, 4.0 mM sodium azide, 1.5 μmoles NADPH, and 1 unit per ml glutathione reductase. The reaction was started by addition of hydroperoxide. Activity was monitored at 340 nm in a Beckman Acta III spectrophotometer maintained at a constant temperature of 37 C by a Haake circulating water bath. Baseline changes (non-specific activity) in optical density of less than 0.002 per min were subtracted from the total change in absorbance per min following the addition of hydroperoxide. The GSHPx activity was expressed as nmoles NADPH oxidized min^{-1} mg $protein^{-1}$.

Since the brain homogenate was contaminated with blood, it was necessary to correct for contributions to the brain GSHPx activity by erythrocytes. The erythrocytes of 15 hospitalized patients were individually isolated by centrifugation at 800 × g for 20 min in an IEC, Model PR 6000 centrifuge, kept at 4 C. The cells were washed twice with 0.9% NaCl and lysed with 0.9% NaCl-0.5% Triton X-100 (1:40). Hemoglobin in each lysed erythrocyte preparation and brain supernatant solution was measured by the method of Drapkin (21). GSHPx activity in these preparations was assayed by the method described above. Sixty μl aliquots were used for Hb and for GSHPx determinations. Enzyme activity in each brain sample was corrected for erythrocyte contribution (average GSHPx activity of the 15 erythrocyte samples) by the following formula: [OD/min (brain)]-[OD/min/mg Hb (RBC) × mg Hb (brain)] = OD/min (brain corrected). GSHPx activity of erythrocytes in these autopsied brain samples ranged from 1-6 nmoles NADPH oxidized/min.

Preparation of hydroperoxides. The fatty acid hydroperoxides were prepared according to the method of Hamberg and Samuelsson (22). The fatty acid (4 mg/ml) was solubilized in 0.019 M NH_4OH. To complete the solubilization, 0.5 ml of 95% EtOH was added (23). Soybean lipoxygenase (1 mg [20000 units/mg] per ml) was dissolved in 0.1 M borate buffer, pH 9.0, and added to the incubation mixture (2.5 ml/4 mg fatty acid). The reaction was allowed to proceed for 30 min at 0 C. Pure oxygen was bubbled throughout the incubation period (23,24). At the end of this time 100 ml each of 95% EtOH and H_2O was added. The mixture was acidified to a pH of less than 3.0 with 5.0 N HCl. Hydroperoxides were extracted 3 times with diethyl ether. The ether was washed 3 times with 0.1 vol H_2O, dried with $MgSO_4$, and evaporated to dryness at room temperature with N_2. Hydroperoxide residue was stored at −20 C in closed containers until analyzed for peroxides (25). The hydroperoxides were solubilized in a mixture 0.1 ml 95% EtOH and 0.1 ml H_2O. A 20 μl aliquot of the appropriate dilutions was used for assay of GSHPx.

In the case of t-butyl and H_2O_2, the solutions were serially diluted with H_2O and a 20 μl aliquot used for the GSHPx assay.

Protein in the 105,000 × g brain supernatant solution was determined by the Lowry technique as modified by Markwell et al. (26), using bovine serum albumin as a standard.

RESULTS

The enzyme, GSHPx, in supernatant solution (105,000 × g) of brain was capable of reducing all 8 lipid hydroperoxides used in the present study (Figs. 1-3). As shown in these figures, these hydroperoxides were reduced at different rates by the brain GSHPx system. The values used in these figures are the means ± S.D. of 3-5 different brain samples claculated by a BMDP statistical analysis program (27) on a Digital computer, Model pdp 11.

In the 18 carbon series saturation of enzyme with linoleic and gamma linolenic was reached with 45 μM and 90 μM of the respective substrate (Fig. 1). At the highest concentration of linolenic (75 μM), the activity was approaching a constant rate with respect to substrate concentrations. These data suggest that hydroperoxides of 18 carbon PUFA, with an unsaturated bond at ω6, saturated the enzyme at the concentrations used.

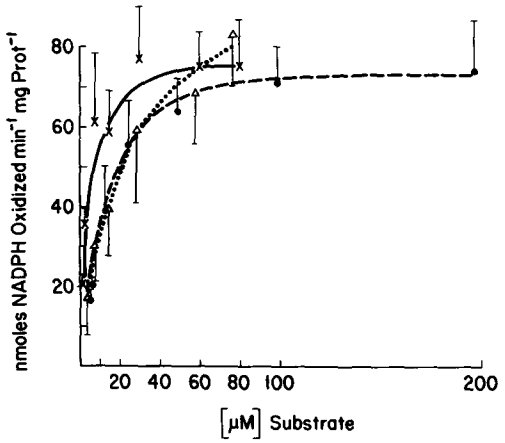

FIG. 1. A plot of GSHPx activity (velocity) in human frontal gray matter versus concentration of peroxides of 18 carbon polyunsaturated fatty acids: X——X, linoleic (18:3ω6) [3]; △·····△, linolenic (18:3ω3) [3], and ●– – – –●, γ linolenic (18:3ω6) [4]. Values are the means obtained with frontal gray matter samples run in duplicate. The number of different brain samples analyzed is indicated in brackets [ ]. The means ±S.D. were calculated by a Digital computer using a BMDP program for statistical analysis (27). The GSH concentration was kept constant at 1.0 mM.

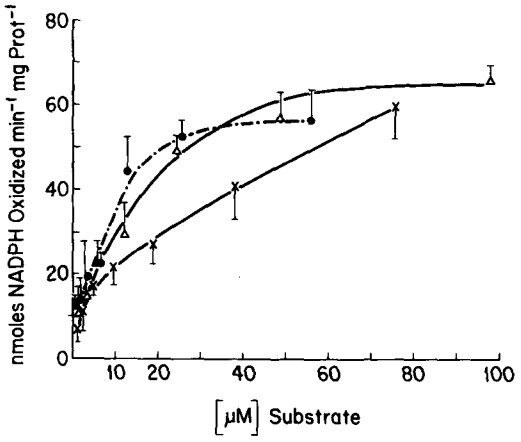

FIG. 2. A plot of GSHPx activity (velocity) in human frontal gray matter versus concentration of peroxides of 20 carbon polyunsaturated fatty acids: X——X, eicosodienoic (20:2ω6); △——△, homo γ linolenic (20:3ω6), and ●–·–·–·–●, arachidonic (20:4ω6). Values are the means obtained with frontal gray matter samples run in duplicate from 5 different human brains. The means ±S.D. were calculated by a Digital computer using a BMDP program for statistical analysis (27). The GSH concentration was kept constant at 1.0 mM.

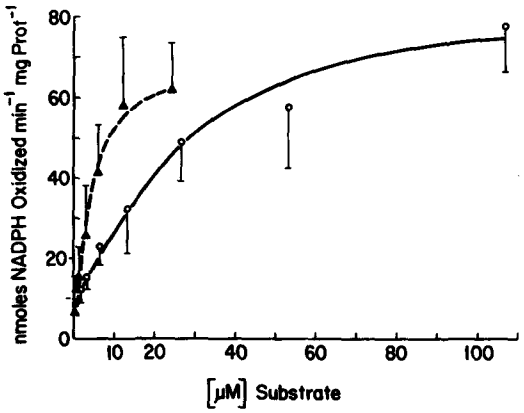

FIG. 3. A plot of GSHPx activity (velocity) in human frontal gray matter versus concentration of peroxides of 22 carbon polyunsaturated fatty acids: ▲—— ——▲, docosotetraenoic (22:4ω6), and ○——○, docosohexaenoic (22:6ω3). Values are the means obtained with frontal gray matter samples run in duplicate from 5 different human brains. The means ±S.D. were calculated by a Digital computer using a BMDP program for statistical analysis (27). The GSH concentration was kept constant at 1.0 mM.

Figures 2 and 3 show the changes in velocity (nmoles NADPH oxidized per min per mg protein) versus substrate concentration when 3 hydroperoxides of the 20 carbon series, namely eicosodienoic, homo gamma linolenic and arachidonic (Fig. 2), and 2 hydroperoxides of the 22 carbon series, docosotetraenoic and docosohexaenoic (Fig. 3) were used as substrates. Homo gama linolenic and arachidonic hydroperoxide saturated the enzyme at 60 and 25 μM, respectively. The substrate, hydroperoxide of eicosodienoic, did not reach a constant rate at the highest concentration tested (75 μM). The two 22 carbon chain length fatty acid hydroperoxides, docosotetraenoic and docosohexaenoic, appeared to saturate the enzyme at 20 and 105 μM, respectively. GSHPx activity with docosohexaenoic hydroperoxide at the highest concentration, was just beginning to approach a constant rate. The other hydroperoxides of the 20 and the 22 carbon PUFA with an unsaturated bond at ω6 had saturated the enzyme except for the hydroperoxide of 11,14 eicosodienoic, a PUFA not generally found in tissues.

Brain GSHPx activity also was examined using H_2O_2 and t-butyl hydroperoxide, the 2 peroxides most commonly used for enzyme assay (Fig. 4). The data indicated that H_2O_2 and t-butyl hydroperoxide saturated the enzyme at 0.1 mM and 0.45 mM, respectively.

In order to compare GSHPx activity using different hydroperoxides as substrates, GSHPx

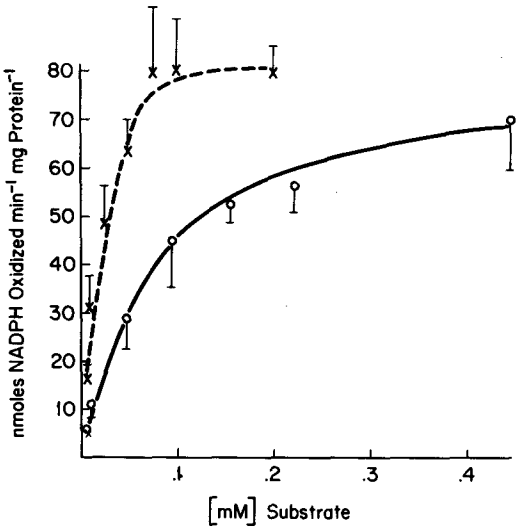

FIG. 4. A plot of GSHPx activity (velocity) in human frontal gray matter versus concentration of hydrogen peroxide and t-butyl hydroperoxide. X– – – –X, H_2O_2 [5], and o——o, t-butyl [4]. Values are the means obtained with frontal gray matter samples run in duplicate. The number of different human brains analyzed is indicated in brackets [ ]. The means ±S.D. were calculated by a Digital computer using a BMDP program for statistical analysis (27). The GSH concentration was kept constant at 1.0 mM.

activity on the linear portion of the curve (Figs. 1-4) was used. A substrate concentration of 0.01 mM was found to be suitable for this comparison, and GSHPx activity (expressed as nmoles NADPH oxidized min^{-1} mg $protein^{-1}$) for the hydroperoxides at 0.01 mM varied from 8-54.7 (Table 1). The hydroperoxide substrates which were reduced most rapidly by GSHPx were gamma linolenic (54.7) and docosotetraenoic (53.0), followed by arachidonic (36.5), linolenic (35.3) and linoleic (32.7). The hydroperoxide substrates which were reduced most slowly by GSHPx were homo gamma linolenic (29.0), docosohexaenoic (29.0) and 11,14 eicosodienoic (22.0). At this concentration of hydroperoxide (0.01 mM), t-butyl hydroperoxide had a GSHPx activity of 8.0 and H_2O_2, 23.5. The natural compounds, lipid hydroperoxide and H_2O_2, were better substrates than the non-natural compound, t-butyl hydroperoxide.

DISCUSSION

GSHPx is a unique enzyme in that it is capable of reducing hydroperoxides of different molecular sizes and shapes, ranging from inorganic (H_2O_2) to organic (PUFA and terpene-type hydroperoxides) (9,12,13). The mechanism of GSHPx reaction is 2 substrate double displacement (ping-pong) (9). Two types of GSHPx, a Se-dependent and a Se-independent, have been identified in the brain (17). Our studies using human brain supernatant solution probably contained both enzymes and glutathione S-transferase, and this group of enzymes was labeled 'brain GSHPx system.' We did not attempt to differentiate the various types of enzymes.

Using this brain GSHPx system, we calculated the apparent Km for each lipoperoxide. In order to use zero order kinetics the second substrate, GSH, was kept constant at 1.0 mM.

TABLE 1

Glutathione Peroxidase Activity Using Different Hydroperoxides

Hydroperoxide	Unsaturation	GSHPx activity (nmoles NADPH oxidized min^{-1} mg $protein^{-1}$)
Linoleic	18:2ω6	32.7
Linolenic	18:3ω3	35.3
γ Linolenic	18:3ω6	54.7
11,14 Eicosodienoic	20:2ω6	22.0
Homo γ Linolenic	20:3ω6	29.0
Arachidonic	20:4ω6	36.5
Docosotetraenoic	22:4ω6	53.0
Docosohexanenoic	22:6ω3	26.5
t-butyl		8.0
H_2O_2		23.5

Enzyme activity in brain 105,000 X g supernatant solution was determined at hydroperoxide concentration of 0.01 mM and GSH concentration of 1.0 mM using the data shown in Figures 1-4. The activity is expressed as NADPH oxidized per min per mg protein and is the mean of 3-5 different human frontal gray matter samples done in duplicate.

The apparent Km was calculated from the slope of a line obtained by a Lineweaver-Burke plot which was drawn by a Digital computer with a BDMP linear regression program (27). Arachidonic hydroperoxide had the lowest apparent Km for the enzyme, 2.1 μM, followed by docosohexaenoic, 3.3 μM, docosotetraenoic, 6.1 μM, 11,14 eicosodienoic, 7.8 μM, and gamma linolenic, 9.0 μM. Hydroperoxides of homo gamma linolenic, 10.9 μM, and linolenic, 15.1 μM appeared to have the lowest affinity for brain GSHPx. An apparent Km of 23.2 mM and 43.4 mM was calculated for H_2O_2 and t-butyl hydroperoxide, respectively. The affinity of lipid hydroperoxides for GSHPx appeared to be higher than that for H_2O_2 and t-butyl. In order to accurately calculate the apparent Km using zero order kinetics, the enzyme and substrate should be purified, and a sufficient amount of the substrate available so the enzyme activity will approach a constant rate with respect to the substrate concentration. In these experiments, however, we were interested primarily in comparing the apparent Km of the different hydroperoxide substrates under similar reaction conditions.

The brain GSHPx system reduced the hydroperoxides of PUFA that have been found in the brain, namely, arachidonic, docosotetraenoic and docosohexaenoic (1,2). These PUFA are present in high concentrations in brain phospholipids (1,2). According to some reports, hydroperoxidation of these phospholipid PUFA yields a product which cannot be reduced by GSHPx (12-14,28). The data of McCay et al. (12-14) suggest that lipid peroxidation is prevented by a radical scavenging protein, a GSH-cytosolic compound, not GSHPx. Their conclusions were based on 2 observations: 1) GSHPx, partially purified, was not able to reduce the microsomal membrane peroxidized phospholipids, and 2) hydroxylated fatty acids could not be isolated and identified by TLC (10). By contrast, Ursini and coworkers (29), using a similar system, observed an increased reduction of fatty acid peroxides at the C-2 position of microsomal membrane phospholipids and suggested that this system included GSHPx, and was not glutathione S-transferase. Further studies on the identification and the mechanisms of action of the cytosolic protein are required before concluding whether GSHPx is involved.

In their paper on human brain GSHPx, Carmagnol et al. (19) reported an activity of 18 nmoles NADPH oxidized min^{-1} mg $protein^{-1}$ using t-butyl hydroperoxide (0.25 mM) as substrate. We obtained a value of 62 nmoles NADPH oxidized min^{-1} mg $protein^{-1}$ using 0.25 mM t-butyl hydroperoxide (Fig. 4). Our values of GSHPx activity in the human brain were higher. This difference may be related to methodology: they used phosphate buffer, pH 7.0, whereas we used Hepes-EDTA, pH 7.5, and they sonicated their tissue and we solubilized the tissue in Triton X-100.

Lipid peroxides in the brain may have an important role in cellular damage (3,7-9) and aging (3-6). DeMarchena et al. (30) had expressed some doubts regarding the significance of brain GSHPx in reduction of hydroperoxides because they were able to detect only small amounts of this enzyme. This concept on lack of significance of GSHPx in reduction of lipid hydroperoxides was supported by McCay et al. (12-14). It is apparent from the data of Prohaska and Ganther (17), Brannan et al. (18) and Carmagnol et al. (19) that the brain contains a significant amount of GSHPx to minimize damage from peroxidation products. Our data indicates that lipid hydroperoxides can be reduced by brain supernatant solution, which we are calling, GSHPx system. In cases of pathological insults, arachidonic acid is released (31, 32) and can be converted to a hydroperoxide (33). These lipid peroxides can activate phospholipase A_2, which causes the release of more unsaturated fatty acids from the C-2 position of phospholipids (34). Excess buildup of these lipid hydroperoxides or decreased enzyme levels and/or activity may cause damage to cell membrane, and thus decrease the brain's ability to function normally. These changes also may be involved in the development of senescence (3-6).

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REFERENCES

1. O'Brien, J.S., and Sampson, E.L. (1965) J. Lipid Res. 6, 545-551.
2. Svennerholm, L. (1968) J. Lipid Res. 9, 570-579.
3. Tappel, A.L. (1978) Adv. Exp. Med. 97, 111-131.
4. Hirai, S.; Okamoto, K., and Morimatsu, M. (1982) in Lipid Peroxides in Biology and Medicine (Yagi, K., ed.) pp. 305-315, Academic Press, New York, NY.
5. Noda, Y.; McGeer, P.L., and McGeer, E.G. (1982) Neurobiol. of Aging 3, 173-178.
6. Harman, D. (1981) Proc. Natl. Acad. Sci. USA, 78, 7124-7128.
7. Pauls, K.P., and Thompson, S.E. (1980) Nature 283, 504-506.
8. Dror, Y.; Budowski, P.; Bupis, J.J.; Sandband, U., and Wolman, M. (1976) Prog. Neuropathol. 3, 343-57.

9. Wolman, M. (1975) Israel J. Med. Scie. Supplement 11, 1-229.
10. Flohe, L., and Gunzler, W.A. (1974) in Glutathione (Flohe, L.; Benohr, H. Ch.; Sies, H.; Waller, H.D., and Wendel, A., eds.) pp. 132-143, Academic Press, New York, NY.
11. Chance, B.; Sies, H., and Boveris, A. (1979) Physiol. Rev. 59, 527-605.
12. McCay, P.B.; Gibson, D.D.; Fong, K-L., and Hornbrook, K.R. (1976) Biochim. Biophys. Acta 431, 459-468.
13. Gibson, D.D.; Hornbrook, K.R., and McCay, P.B. (1980) Biochim. Biophys. Acta 620, 572-582.
14. McCay, P.B.; Gibson, D.D., and Hornbrook, K.R. (1981) Fed. Proc. 40, 199-205.
15. Little, C. (1972) Biochim. Biophys. Acta 284, 375-381.
16. Christopherson, B.O. (1968) Biochim. Biophys. Acta 164, 36-46.
17. Prohaska, J.R., and Ganther, H.E. (1976) J. Neurochem. 27, 1379-1387.
18. Brannan, T.S.; Maker, H.S.; Weiss, C., and Cohen, G. (1980) J. Neurochem. 35, 1013-14.
19. Carmagnol, F.; Sinet, P.M., and Jerome, H. (1983) Biochim. Biophys. Acta 759, 49-57.
20. Beutler, E. (1975) in: Whole Red Cell Metabolism. A Manual of Biochemical Methods (Beutler, E., ed.) pp. 71-73, 2nd ed., Grune & Stratton, New York, NY.
21. Drapkin, D. (1967) in Clinical Hematology (Wintrobe, M.M., ed.) p. 403, Lea and Febiger, Philadelphia, NY.
22. Hamberg, M., and Samuelsson, B. (1967) J. Biol. Chem. 242, 5329-5335.
23. Funk, M.O.; Isaac, R., and Porter, N.A. (1976) Lipids 11, 113-117.
24. Streckert, G., and Stan, J-J. (1975) Lipids 10, 847-854.
25. Asakawa, T., and Matsushita (1980) Lipids 15, 965-67.
26. Markwell, M.A.K.; Haas, S.M.; Bieber, L.L., and Tolbert, N.E. (1978) Anal. Biochem. 87, 206-210.
27. Biomedical Computer Programs (1979) (Dixon, W.J., and Brown, M.B., eds.) Univ. California Press, Berkeley, CA.
28. Grossman, A., and Wendel, A. (1983) Eur. J. Biochem. 135, 549-552.
29. Ursini, F.; Maiorino, M.; Valente, M.; Ferri, L., and Gregolin, C. (1982) Biochim. Biophys. Acta 710, 197-211.
30. DeMarchena, O.; Guarnieri, M., and McKhann, G. (1974) J. Neurochem. 22, 773-776.
31. Gardiner, M.; Nilsson, B.; Rechncrona, S., and Siesjö, B.K. (1980) J. Neurochem. 36, 1500-1505.
32. Bazan, N.G. (1970) Biochim. Biophys. Acta 218, 1-10.
33. Chan, P.H.; Yurko, M., and Fishman, R.A. (1982) J. Neurochem. 38, 525-531.
34. Van Den Bosch, H. (1980) Biochim. Biophys. Acta 604, 191-246.

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Analysis of Autoxidized Fats by Gas Chromatography-Mass Spectrometry. IX. Homolytic vs. Heterolytic Cleavage of Primary and Secondary Oxidation Products

E.N. FRANKEL*, W.E. NEFF and **E. SELKE**, *Northern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Illinois 61604*

ABSTRACT

To elucidate the genesis of volatile lipid oxidation products, thermal homolytic and acid heterolytic decomposition processes were compared. Secondary oxidation products were decomposed thermally (200 C), and the volatiles formed were identified by capillary gas chromatography-mass spectrometry (GC-MS). Oxidation products also were decomposed in the presence of HCl-methanol, and the resulting dimethyl acetals were identified by GC-MS. The volatile thermal decomposition products were those expected by homolytic β-scission on both sides of the hydroperoxide group. No dialdehydes were identified under our thermal decomposition conditions. In contrast, the acetals formed by acid decomposition were those expected by selective heterolytic scission between the hydroperoxide group and the allylic double bond. Dialdehydes identified from acid decomposition of cyclic peroxides and dihydroperoxides included malonaldehyde and 2,4-hexadienedial.
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INTRODUCTION

Previous papers in this series (1,2) reported the identification of volatile thermal decomposition products of hydroperoxy cyclic peroxides. These papers provided direct evidence for the contribution of secondary lipid oxidation products as precursors of volatiles. Many of these volatile lipid oxidation products are associated with flavor deterioration of lipids (3), with cellular damage (4,5), and with a decrease in the safety of fat-containing foods (6). A major part of the volatile thermal decomposition products are those expected by the generally recognized mechanism involving homolytic β-scission on both sides of the hydroperoxide group (3). In another paper (7), we described an acetalation-acid decomposition procedure to investigate the formation of malonaldehyde from primary and secondary products of lipid oxidation. A heterolytic cleavage mechanism was suggested to explain the fragmentation of hydroperoxy cyclic peroxides and 1,3-dihydroperoxides to produce malonaldehyde as the tetramethyl acetal derivative.

This paper describes an extension of our studies on the thermal decomposition volatile products of other recently identified secondary products, including dihydroperoxides and hydroperoxy bicycloendoperoxides (1,8,9). The dimethyl acetal cleavage products resulting from decomposition with HCl-methanol also were identified by gas chromatography-mass spectrometry (GC-MS) and compared to the corresponding thermal decomposition products generated directly on the injector port of a gas chromatograph.

* To whom correspondence should be addressed.

EXPERIMENTAL

Monohydroperoxides, dihydroperoxides, hydroperoxy epidioxides, and hydroperoxy bicycloendoperoxides were prepared from oxidized methyl linoleate and linolenate by a general procedure developed previously (1,8-10), which involves the following steps: (a) autoxidation (8) or photosensitized oxidation (1,9); (b) silicic acid column chromatographic separation of monohydroperoxides from secondary oxidation products by elution with mixtures of diethyl ether/hexane (11); (c) high-pressure liquid chromatography (HPLC) of dihydroperoxides and hydroperoxy bicycloendoperoxides on a 6 μ microporous silica column (Zorbax Sil, 250 × 2.12 cm, Dupont, Wilimington, Delaware) eluting with a mixture of absolute ethanol/hexane (3:9, v/v), and hydroperoxy epidioxides on a 10 μ microporous silica column (Partisil-10 M9, 100 × 0.94 cm, Whatman, Clifton, New Jersey) eluting with a mixture of hexane/methylene chloride/ethyl acetate (7:4:1, v/v/v) (9); and (d) thin-layer chromatography (TLC) of each chromatographic fraction to check for functional purity (1,9).

Thermal decomposition of oxidation pro-

ducts was achieved in the injector port of a GC-MS system at 200 C, using a capillary fused silica column bonded with Durabond DB-5 (J&W Scientific, Rancho Cordova, Califorina) (2). Acid decomposition was carried out with HCl-methanol by the same procedure reported for the formation of malonaldehyde (7). The resulting dimethyl acetal derivatives were analyzed by GC-MS, using a glass column packed with 10% SP 2330 (Supelco, Inc., Bellefonte, Pensylvania) (7). Thermally generated volatiles were identified as before with a computerized GC-MS system (12,13), matching mass spectra with those of our reference library and confirming by GC retention data. Acid-generated dimethyl acetals were identified by comparing their mass spectra and GC retention times with those of dimethyl acetals of authentic aldehydes (Bedoukian Research, Inc., Danbury, Connecticut; Aldrich Chemical Co., Milwaukee, Wisconsin; Eastman Organic Chemicals, Rochester, New York; K & K Laboratories, Long Island City, New York). Methyl 9-oxononanoate was prepared by reductive ozonolysis of methyl oleate (14). When reference compounds were not available, the identification was based on mass spectral interpretation from the literature (15,16).

RESULTS AND DISCUSSION

Thermal Decomposition Products

Dihydroperoxides were formed in relatively significant amounts (1.0 to 5.6%) and hydroperoxy bicycloendoperoxides in only minor amounts (0.1 to 0.3%) in our samples of photosensitized oxidized methyl linolenate (9). The volatiles formed by thermal decomposition of these secondary oxidation products were identified by capillary GC-MS (Table 1). Propanal was an important volatile produced from the oxidation products containing a 16-hydroperoxide group: 9,16(I)-, 10,16(II)-, 9,12+13, 16(V)-dihydroperoxides, and from the 9- and 16-hydroperoxy bicycloendoperoxide mixture (VI). Other significant volatiles included 2-butenal from dihydroperoxide IV, methyl octanoate from dihydroperoxides I, IV and V, methyl 9-oxononanoate from dihydroperoxides I, III, IV and V and from hydroperoxy bicycloendoperoxides VI, methyl 10-oxo-8-decenoate from dihydroperoxides II and III, methyl

TABLE 1

Capillary GC-MS Analysis[a] of Volatiles from Thermally Decomposed Dihydroperoxides (I-V) and Hydroperoxy Bicycloendoperoxides (VI) (Schemes 1-4)[b]

Volatile compounds	Relative retention	Relative peak area per cent					
		I	II	III	IV	V	VI
Acetaldehyde	0.11	0.6	0.1	0.9	1.9	0.2	0.3
Ethane	0.12	1.0	0.4	1.0		1.2	0.9
Acetone	0.12	3.1	4.3	3.8	4.8	5.8	3.1
Propanal	0.13	19.4	24.7		2.1	19.9	26.0
Butanal	0.16		0.6	1.2		2.4	2.0
2-Butenal	0.17	1.8	2.0	2.1	29.1	1.7	1.6
1,4-Hexadiene	0.24			0.9	1.9	3.3	
2,4-Hexadienal[c]	0.58-0.61			0.6	1.0	0.4	
Me Hexanoate	0.65		0.2	0.7	0.1	0.3	
Butyl Furan[c]	0.72						5.8
2-Heptenal	0.74					0.7	
2,4-Heptadienal[c]	0.80-0.81	0.2	1.3	4.6	0.7	1.2	5.5
Me Heptanoate	0.83	0.1	1.0	1.9	0.9	1.0	0.2
Me Octanoate	1.00	7.8	0.8	3.5	10.0	10.0	2.5
Me 8-Oxooctanoate	1.35	0.6	1.9	3.0	1.8	2.6	0.3
Me 9-Oxononanoate	1.53	38.8	2.2	7.1	9.2	9.8	30.3
Me 10-Oxodecanoate	1.63	0.6	0.3	6.1	0.8	1.0	2.0
Me 10-Oxo-8-decenoate	1.71	2.7	40.9	35.6	5.1	5.0	2.0
Me Furan octanoate[d]	1.73				5.0	7.6	1.2
Me 11-Oxo-9-undecenoate[d]	1.84	4.1	4.4	5.5	7.0	6.5	0.9
Me 12-Oxo-8,10-dodecadienoate[c,d]	1.94-1.96			3.7			
Me 13-Oxo-9,11-tridecadienoate[c,d]	2.15-2.19				7.8	5.5	1.8
Unidentified		19.2	14.9	17.8	10.8	13.9	13.6

[a]Quantitation based on flame ionization detection.

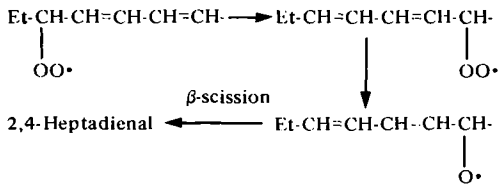
[b]Compound I was a mixture of *cis,trans,trans-* and *trans,trans,trans*-9,16-dihydroperoxides; II-IV were pure positional isomers; V was a mixture of 9,12 (VA) and 13.16 (VB) dihydroperoxides; VI was a mixture of 9, (VIA) and 16 (VIB) hydroperoxy bicycloendoperoxides. All compounds were mixtures of diastereomers.

[c]Separated *cis,trans* and *trans,trans* isomers are combined.

[d]Tentative identification (2).

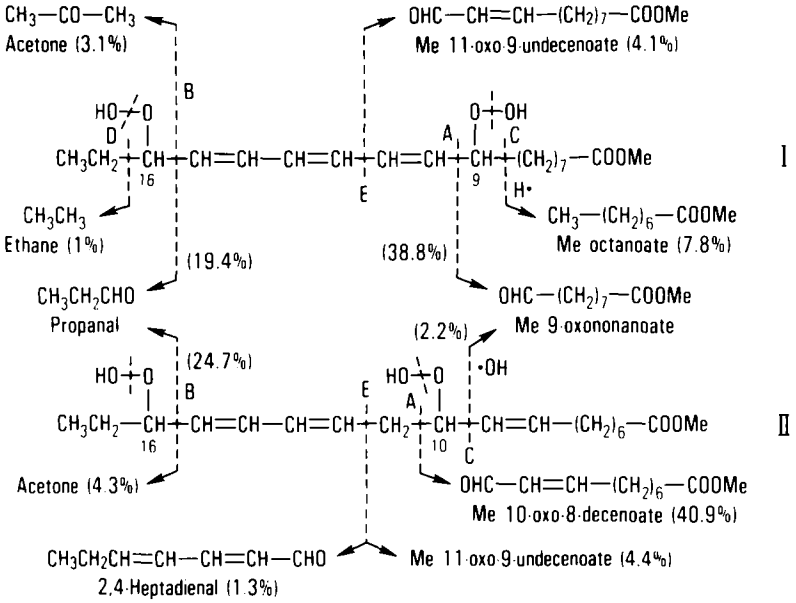
furan octanoate from dihydroperoxides IV and V, and methyl 11-oxo-9-undecenoate from dihydroperoxides III, IV and V.

Fragmentation Schemes 1-4 account for 65 to 80% of the total volatiles produced by thermal decomposition of the dihydroperoxides I to V and hydroperoxy bicycloendoperoxides VI (Table 1). More detailed mechanisms to explain formation of major volatile products from the multitude of reactions indicated in these schemes have been discussed previously (1-3). In the 9,16- and 10,16-dihydroperoxides (I and II), internal cleavage A producing aldehyde esters and internal cleavage B producing propanal are the most important (Scheme 1). External cleavage C, on the other side of the first hydroperoxide group, explains the formation of methyl octanoate in I involving the addition of an H• radical, as well as the formation of methyl 9-oxononanoate in II involving the addition of an •OH radical followed by rearrangement (2,13). Internal cleavage E may explain the formation of methyl 11-oxo-9-undecenoate in I by an allylic rearrangement of oxygen. The formation of methyl 11-oxo-9-undecenoate in II by cleavage E would involve rearrangment of O• and H• radicals. The formation of 2,4-heptadienal can be similarly rationalized by a rearrangement via a peroxy radical intermediate:

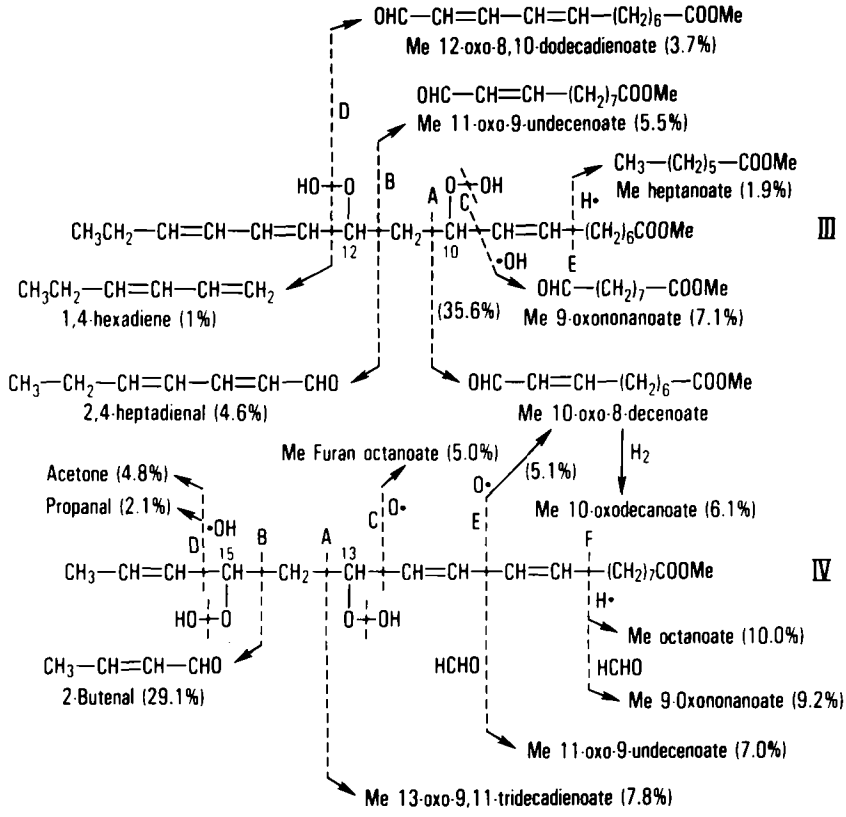


The formation of acetone usually is associated with the formation of propanal (see I, II, IV, VB and VIA, Schemes 1-4). Oxygen migration from carbon-1 to carbon-2 may be postulated via an epoxy intermediate (17), as previously was suggested for the conversion of 2-octanone into octanal (2). However, this mechanism would not apply to 10,12-dihydroperoxide III, which produced acetone without the formation of propanal.

In the fragmentations shown in Scheme 2, cleavage A apparently was more important in 10,12-dihydroperoxide (III) than in 13,15-dihydroperoxide (IV), and the reverse was true for cleavage B. This difference may be due to the large number of fragments produced on the right side of cleavage A in IV (C, E and F totaling 36.3%), compared to those in III (C and E totaling 9.0%). Alternatively, cleavage products containing a conjugated diene system may be less important because they are more reactive and can undergo further oxidative



SCHEME 1

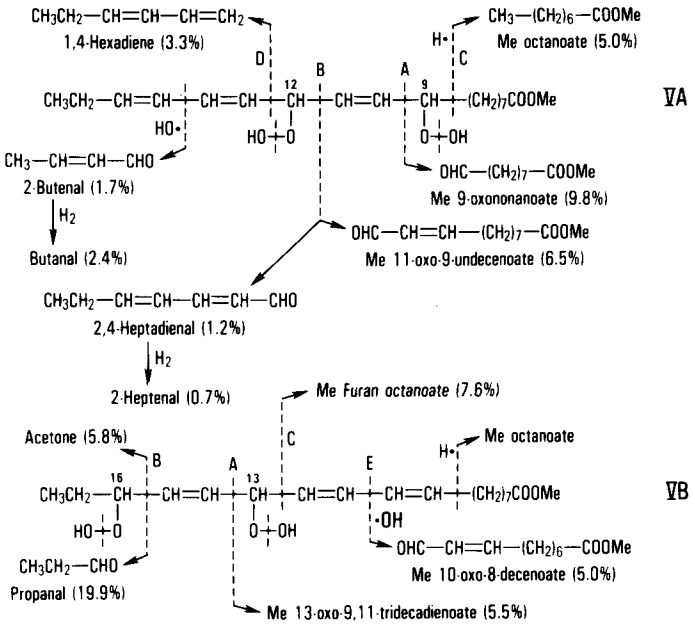


SCHEME 2

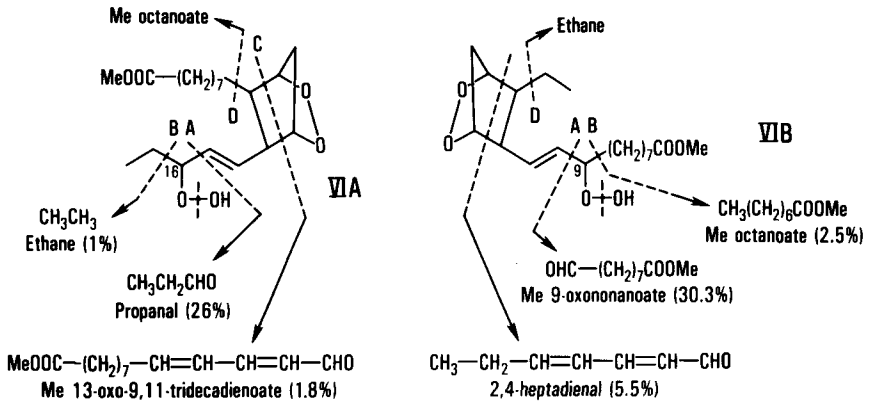
degradation. 2,4-Heptadienal from III and methyl 13-oxo-9,11-tridecadienoate from IV can be further oxidized and cleaved into shorter aldehydes in the same way as previously reported with 2,4-decadienal (3,18). The formation of methyl 11-oxo-9-undecenoate by cleavage B in III can be rationalized by rearrangement via a 3-carbon allylic intermediate, as suggested for dihydroperoxide I. Methyl 11-oxo-9-undecenoate from IV may be formed either by cleavage E with addition of formaldehyde (2) or by oxidative degradation of the conjugated diene precursor, methyl 13-oxo-9, 11-tridecadienoate.

The formation of methyl furan octanoate [methyl 8-(2-furyl-)octanoate] by cleavage C in dihydroperoxide IV is equivalent to the fragmentation previously observed with 16-hydroperoxy-13,15-epidioxy-9,11-octadecadienoate (2). The formation of malonaldehyde would be expected by cleavages C and D in both dihydroperoxides III and IV. However, this dialdehyde apparently is too unstable under our conditions of thermal decomposition on the GC injector port. Indeed, malonaldehyde was not observed previously as one of the thermal decomposition products expected from the 5-member cyclic peroxides of oxidized methyl linolenate (2). On the other hand, the dimethyl acetal of malonaldehyde was detected as an important product of the decomposition of dialdehydes III and IV under milder acid conditions (7) (see Acid Decomposition Products).

Fragmentations of 1,4-dihydroperoxides VA and VB (Scheme 3) are similar to those of the 1,3-dihydroperoxides III and IV (Scheme 2). Cleavage B in VA and Cleavage A in VB are less important because they are associated with the formation of unstable products containing conjugated diene systems (2,4-heptadienal in VA and methyl 13-oxo-9,11-tridecadienoate in VB). The formation of 2-butenal and butanal can be rationalized from VA either by cleavage between C-14 and C-15 (Scheme 3) or by oxidative degradation of 2,4-heptadienal produced by cleavage B. Methyl 10-oxo-8-decenoate from VB can be explained similarly either by cleavage E between C-10 and C-11 or by oxidative degradation of methyl 13-oxo-9,



SCHEME 3



SCHEME 4

11-tridecadienoate. Methyl furan octanoate is formed by cleavage C and addition of oxygen radical in the same way as in dihydroperoxide IV (Scheme 2).

Thermal fragmentation of the hydroperoxy bicycloendoperoxides shows dominant cleavage A between the hydroperoxide group and the allylic double bond to form propanal in VIA and methyl 9-oxononanoate in VIB (Scheme 4). Cleavage B on the other side of the hydroperoxide group and cleavage C across the endoperoxide ring explain the formation of methyl 13-oxo-9,11-tridecadienoate in VIA and 2,4-heptadienal in VIB. Cleavage C across the ring apparently is more important than cleavage D on the side of the endoperoxide ring, producing methyl octanoate in VIA and ethane in VIB. Cleavage C also would be expected to form malonaldehyde as suggested by Pryor et al. (19,20), but this dialdehyde was again too unstable to detect under our thermal decomposition conditions. However, malonaldehyde was detected as a product of VIA and VIB under acid decomposition conditions (cf. below).

Acid Decomposition Products

Our successful identification of malonal-

dehyde, as the tetramethyl acetal, by treating various lipid oxidation products with a dilute HCl-methanol reagent (7) prompted us to study the products obtained under the milder acid conditions in relation to those of thermal decomposition at 200 C. The aldehyde fragmentation products obtained under acid conditions were identified by MS as the dimethyl acetals by comparison with a series of reference aldehydes (Table 2). The MS characteristics of these derivatives generally agreed with those reported for higher aldehydes (16), with significant mass fragments for M-OCH_3, M-$HOCH_3$, M-(OCH_3 + $HOCH_3$), M-$(CH_3O\text{-}CH\text{-}OCH_3)^+$, $(CH_3O\text{-}CH\text{-}OCH_3)^+$ and $CH_2{=}CH\text{-}CH{=}\overset{+}{O}CH_3$.

We first examined the fragmentation of monohydroperoxides with HCl-methanol to obtain a better understanding of the decomposition pathways of secondary lipid oxidation products. Table 3 compares the GC-MS analysis of aldehydes (as the dimethyl acetals) produced by acid decomposition from pure hydroperoxides of autoxidized methyl linoleate (9- + 13-OOH) with those from photosensitized oxidized methyl linoleate (9- + 10- + 12- + 13-OOH). Although a greater proportion of peaks were unidentified in autoxidation-derived hydroperoxides, they produced significantly more hexanal and methyl 9-oxononanoate and less 3-nonenal and methyl 12-oxo-9-dodecenoate than the photosensitized oxidation-derived

TABLE 2

Mass Spectral Data of Aldehyde Dimethyl Acetals

Aldehyde Di Me Acetals	Mol weight	Relative abundance of structurally significant masses[a], %						Other base peak
		M-31	M-32	M-63	M-75	75	71	
3-Hexenal	146	55	–	36	100	74	100	–
Heptanal[b]	160	11	1	10	1	100	12	–
2-Heptenal[b]	158	48	8	11	–	37	100	–
2,4-Heptadienal[b]	156	87	40	73	80	22	7	40
2-Nonenal[b]	186	27	7	1	3	–	55	40
2,4-Nonadienal[b]	184	14	7	8	5	51	41	40
2,6-Nonadienal	184	1	4	1	2	100	7	–
3,6-Nonadienal	184	1	2	3	3	100	3	–
2,4-Decadienal[b]	198	36	46	4	69	23	21	45
Me 9-oxononanoate	232	49	1	20	2	100	62	–
Me 12-oxo-9-dodecenoate	272	2	6	3	–	100	20	–
Me 15-oxo-9,12-pentadecadienoate	312	18	72	11	1	96	28	84
Propanedial	164	0.4	0.5	8	1	100	3	–
2,4-Hexadienedial	202	4	–	–	4	100	3	–

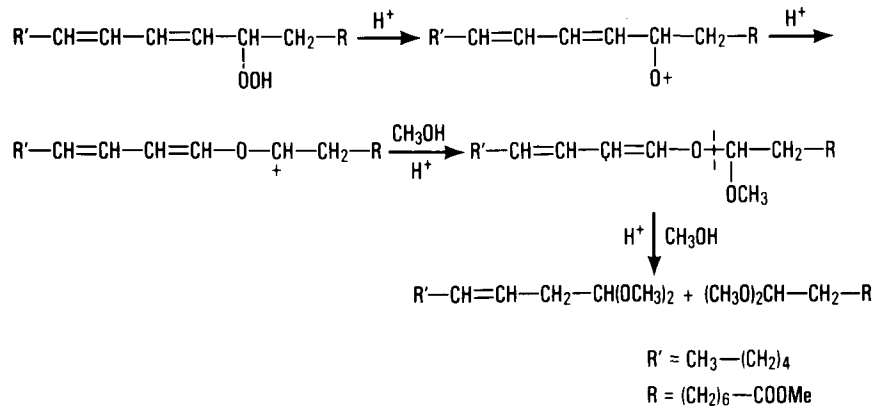
[a]31=OCH_3; 32=$HOCH_3$; 63=OCH_3 + $HOCH_3$; 75=$(CH_3O\text{-}CH\text{-}OCH_3)^+$; 71=$CH_2{=}CH\text{-}CH{=}\overset{+}{O}CH_3$ (16).
[b]Reference compounds purchased commercially.

TABLE 3

GC-MS Analysis[a] of Dimethyl Acetals from Acid Decomposed Monohydroperoxides[b] of Methyl Linoleate

Aldehydes (Di Me Acetals)	Relative retention[c]	Relative peak area per cent (origin)[d]	
		Autoxidation	Photosensitized oxidation
Hexanal	1.18	17 (13-OOH)	12 (13-OOH)
3-Nonenal	1.58	2 (9-OOH)	4 (9- + 10-OOH)
Me 9-Oxononanoate	2.50	42 (9-OOH)	30 (9- + 10-OOH)
Me 12-Oxo-9-dodecenoate	2.88	21 (13-OOH)	51 (12- + 13-OOH)
Unidentified		18	3

[a]Quantitation based on flame ionization detection.
[b]Mixtures of geometric (*cis, trans* and *trans, trans*) and positional (9 and 13) hydroperoxides.
[c]Relative to n-tridecane (7).
[d]Based on Scheme 5.



SCHEME 5

hydroperoxides. This difference in distribution of cleavage products can be explained clearly by the presence of 21-25% of 10- and 12-isomers (21,22) in the hydroperoxides from photosensitized oxidation of methyl linoleate. In both types of hydroperoxides, the major volatile products are those derived exclusively from heterolytic cleavage between the hydroperoxide group and the allylic double bond (Scheme 5). This heterolytic cleavage mechanism to produce dimethyl acetals of aldehydes is essentially the same as that previously proposed for the oxidation of methyl oleate and linoleate in an HCl-methanol system (23, 24). In contrast to this heterolytic mechanism, the homolytic mechanism recognized for the thermal decomposition of hydroperoxides (3) involves cleavage on both sides of the hydroperoxide group and produces a much more complex mixture of volatile materials (13) than under the acid conditions used in the present work. It is interesting to note that ether acids similar to the ether intermediates in Scheme 5 have been reported as enzyme products in potatoes and have been decomposed by acid hydrolysis into unsaturated aldehydes and oxo acids (25).

The GC-MS analyses of aldehydes (as dimethyl acetals) from monohydroperoxides, dihydroperoxides, hydroperoxy epidioxides and hydroperoxy bicycloendoperoxides of methyl linolenate are compared in Table 4. The monohydroperoxides of autoxidized linolenate (9- + 12- + 13- + 16-OOH) produced the same aldehydes as the respective hydroperoxides of phtotosensitized oxidized linolenate (9- + 10- + 12- + 13- + 15- + 16-OOH). The hydroperoxides derived by autoxidation produced more methyl 12-oxo-9-dodecenoate and less propanal and 3.6-nonadienal than the hydroperoxides derived by photosensitized oxidation. With both hydroperoxides the decomposition products obtained with HCl-methanol are those expected by selective cleavage between the hydroperoxide group and the allylic double bond (Scheme 5).

The acid cleavage products from 9,16-dihydroperoxide (I) of linolenate include propanal, methyl 9-oxononanoate in major amounts and 2,4-hexadienedial in minor amounts (Table 4). These products are expected by the heterolytic mechanism we previously suggested for the formation of malonaldehyde (propanedial) from 1,3-dihydroperoxides (7). Selective cleavage A and B occur on the internal bonds between each hydroperoxide group and allylic double bonds (Scheme 6). The center dicarbonyl fragment, 2,4-hexadienedial, was detected only in small quantities, apparently because its stability is lower than that of the end saturated monocarbonyl derivatives. Acid fragmentation of 10,16-dihydroperoxide (II) proceeded the same way as I, producing the same aldehydes. Methyl 9-oxononanoate from II was produced by an A cleavage between C-9 and C-10, because the double bond is located between C-8 and C-9 (Scheme 1). The other fragmentation proceeded as depicted in Scheme 6 for the 9,16-dihydroperoxide (I) by selective cleavage A and B.

Aldehyde fragments of 10,12-dihydroperoxide (III) (3-hexenal and methyl 9-oxononanoate) and of 13,15-dihydroperoxide (IV) (propanal and methyl 12-oxo-9-dodecenoate) (Table 4) are those expected by cleavage on the outside of each hydroperoxide group because of the allylic double bonds (cleavages C and D in Scheme 2). This type of heterolytic cleavage was suggested previously for the formation of malonaldehyde from dihydroperoxides III and IV (7), and explains the formation of this dialdehyde from dihydroperoxides III to V and

TABLE 4

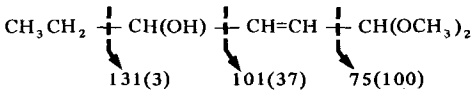
GC-MS Analysis[a] of Dimethyl Acetals from Acid Decomposition of Methyl Linolenate Monohydroperoxides and Secondary Products (Schemes 1-4,7)

Aldehydes (Dimethyl acetals)	Relative retention[b]	Relative peak area per cent (origin)[c]								
		Monohydroperoxides[d]		Dihydroperoxides[e]					Hydroperoxy	
									Epidioxides[f]	Bicyclics[e]
		Auto	Photo	I	II	III	IV	V[g]	VII	VI
Propanal	0.95	16 (16-OOH)	29 (15- + 16-OOH)	38	30		52	42 (16-OOH)	4 (16-OOH)	36 (16-OOH)
Propanedial	1.11					1	1	0.2	1	1
3-Hexenal	1.21	1 (12- + 13-OOH)	0.4 (12- + 13-OOH)			4		1	1 (9-OOH)	1 (9-OOH)
2,4-Hexadienedial	1.46			2	1					
2,4-Heptadienal	1.67								7 (9-OOH)	
3,6-Nonadienal	2.03	3.6 (9-OOH)	14 (9- + 10-OOH)							
Me 9-oxononanoate	2.50	17 (9-OOH)	20 (9- + 10-OOH)	49	60	80		42 (9-OOH)	57 (9-OOH)	39 (9-OOH)
Me 12-oxo-9-dodecenoate	2.88	20 (12- + 13-OOH)	7 (12- + 13-OOH)				32	2 (12-OOH)	4 (16-OOH)	2 (16-OOH)
Me 13-oxo-9,11-tridecadienoate	3.00								4 (16-OOH)	
Me 15-oxo-9,12-pentadecadienoate	3.20	18 (16-OOH)	25 (15- + 16-OOH)							
Unidentified peaks		24	5	11	9	15	15	12.4	22	21

[a,b]See footnotes in Table 3.
[c]Based on Schemes 5-7.
[d]See footnote b in Table 3.
[e]See footnote b in Table 1.
[f]Mixture of positional 9 and 16-hydroperoxy epidioxides and corresponding diastereomers.
[g]Includes 0.4% dimethyl acetal of 4-hydroxy-2-hexenal (see identification in text).

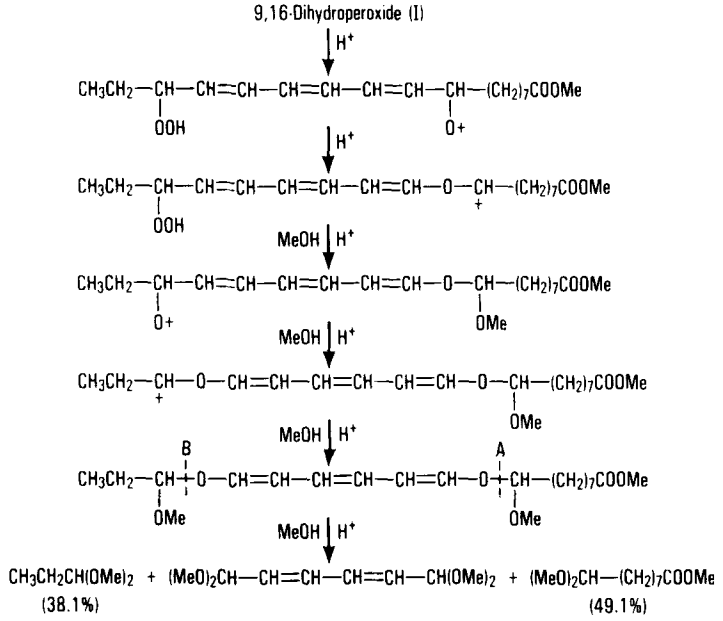
cyclic peroxides VI and VII (Table 4).

The principal aldehydes produced from the mixture of 9,12- and 13,16-dihydroperoxides (V) included propanal from cleavage of the 16-hydroperoxide group in VB, and methyl 9-oxononanoate from cleavage of the 9-hydroperoxide group in VA (Table 4). A small amount of methyl 12-oxo-9-dodecenoate came from cleavage between C-12 and C-13 in VB by the heterolytic mechanism depicted in Scheme 5. By this mechanism, cleavage D in VA and cleavage A in VB produced small amounts of 3-hexenal and methyl 13-oxo-9,11-tridecadienoate (Scheme 3, dimethyl acetal products), respectively. The dimethyl acetal of 4-hydroxy-2-hexenal expected from VB by cleavage C (Scheme 3) was identified by the characteristic ions, m/z (relative %): M-31, 129 (30); M-32, 128 (4); M-63, 97 (20); M-75, 85 (2), 75 (53), 71 (100), and the following fragmentation:

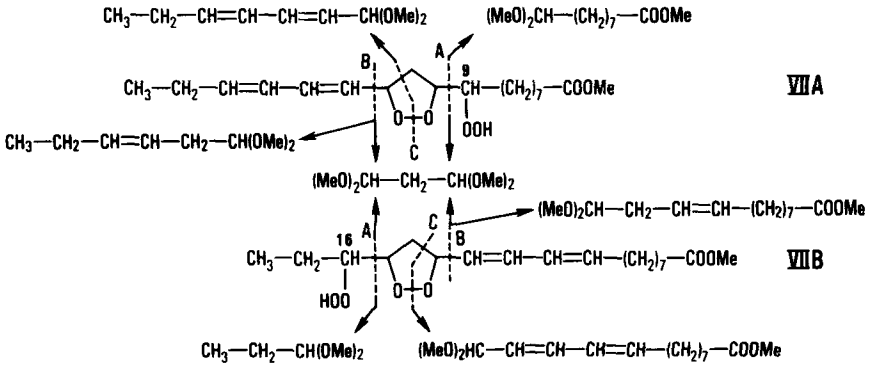


This type of hydroxy aldehyde belongs to a series of aldehydes reported to have significant biological activity (26).

The aldehyde products from the mixture of 9- and 16-hydroperoxy epidioxides VII (Table



SCHEME 6



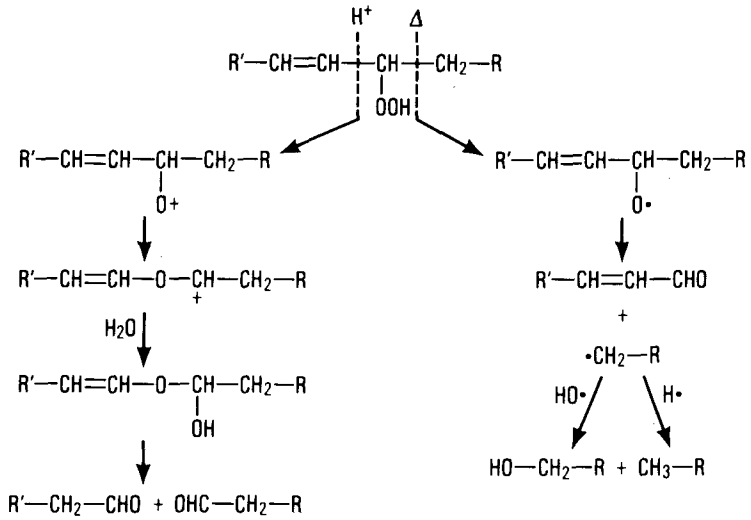
SCHEME 7

4) can be explained by Scheme 7. The formation of malonaldehyde (as the tetramethyl acetal) was explained previously by a heterolytic mechanism, in which the cyclic peroxide cleaves like a monohydroperoxide in forming an ether intermediate allylic to the double bond (7). In addition to this pathway, the formation of 2,4-heptadienal from VIIA and of methyl 13-oxo-9,11-tridecadienoate from VIIB requires peroxide ring cleavage C.

The main acid cleavage products of the hydroperoxy bicycloendoperoxides included propanal from the 16-hydroperoxy isomer VIA and methyl 9-oxononanoate from the 9-hydroperoxy isomer VIB (Table 4). The formation of these products is expected by the same heterolytic ruptures A and B depicted for the corresponding 9,16-dihydroperoxide I (Scheme 6). Methyl 12-oxo-9-dodecenoate derived from VIA and 3-hexenal from VIB, identified in small amounts as the dimethyl acetal derivatives, correspond to the central fragments remaining after formation of malonaldehyde and heterolytic ruptures between the hydroperoxides and allylic double bonds.

The acid heterolytic mechanism involving selective rupture between the hydroperoxide group and allylic double bond previously investigated with simple monohydroperoxides (23,24,27,28) has been confirmed in this study, with both primary and secondary oxidation products of methyl linolenate. In contrast to this straightforward heterolytic mechanism, the thermal homolytic mechanism involves an alkoxy intermediate that cleaves on each side of the hydroperoxide group (3,13). With allylic hydroperoxides, this homolytic β-scission would produce an alkyl radical (R•) on one side and a vinyl radical (R′-CH=CH•) on the other side of the hydroperoxide group. The vinylic radical may be an unlikely intermediate, because the weaker bonds to the alkyl group would be more easily dissociated (29).

Another thermolytic scheme known as the Hock cleavage has been recognized and generally accepted (30,31) because it does not invoke unstable vinylic radicals. Hock cleavage is related to the selective heterolytic mechanism in Scheme 5 and produces the same aldehydes by hydrolysis (3). It is clear, however, that the greater complexity of thermal decomposition products, including hydrocarbons, alcohols, ketones and esters (3,13), is due to cleavage on both sides of the hydroperoxide group. To explain the formation of cleavage products other than aldehydes, a "mixed" homolytic-heterolytic type mechanism may be postulated to avoid the formation of unfavorable vinylic radicals (Scheme 8). By this mechanism, β-scission would occur homolytically on the alkyl side of the hydroperoxide to produce 2-alkenals, and heterolytically on the unsaturated side of the hydroperoxide to produce alkanals. The remaining free radical fragments now can lead to hydrocarbons, alcohols and lower esters. This mechanism is supported by the known acidity of hydroperoxides reported to have higher acid strengths than the corresponding alcohols (32).



SCHEME 8

ACKNOWLEDGMENT

We thank Ned A. Porter, Duke University, Durham, North Carolina; William A. Pryor, Louisiana State University, Louisiana and Cecil R. Smith, NRRC, for helpful discussions.

REFERENCES

1. Frankel, E.N.; Neff, W.E.; Selke, E., and Weisleder, D. (1982) Lipids 17, 11-18.
2. Frankel, E.N.; Neff, W.E., and Selke, E. (1983) Lipids 18, 353-357.
3. Frankel, E.N. (1983) Prog. Lipid Res. 22, 1-33.
4. Pryor, W.A. (1978) Photochem. Photobiol. 28, 787-801.
5. Tappel, A.L. (1980) in Free Radicals in Biology (Pryor, W.A., ed.) Vol. IV, pp. 2-47, Academic Press, New York.
6. Simic, M.G., and Karel, M., editors. (1980) Autoxidation in Foods and Biological Systems, Plenum Press, New York.
7. Frankel, E.N., and Neff, W.E. (1983) Biochim. Biophys. Acta, 754, 264-270.
8. Neff, W.E.; Frankel, E.N., and Weisleder, D. (1981) Lipids 16, 439-448.
9. Neff, W.E.; Frankel, E.N., and Weisleder, D. (1982) Lipids 17, 780-790.
10. Neff, W.E.; Frankel, E.N.; Scholfield, C.R., and Weisleder, D. (1978) Lipids 13, 415-421.
11. Gardner, H.W. (1970) J. Lipid Res. 11, 311-321.
12. Selke, E.; Rohwedder, W.K., and Dutton, H.J. (1977) J. Am. Oil Chem. Soc. 54, 62-67.
13. Frankel, E.N.; Neff, W.E., and Selke, E. (1981) Lipids 16, 279-285.
14. Adlof, R.O.; Neff, W.E.; Emken, E.A., and Pryde, E.H. (1977) J. Am. Oil Chem. Soc. 54, 414-416.
15. McFadden, W.H.; Wasserman, J.; Corse, J.; Lundin, R.E., and Teranishi, R. (1964) Anal. Chem. 36, 1031-1037.
16. Christiansen, K.; Mahadevan, V.; Viswanathan, C.V., and Holman, R.T. (1969) Lipids 4, 421-427.
17. Beckwith, A.L.J., and Ingold, K.U. (1980) in Rearrangements in Ground and Excited States (de Mayo, P., ed.) Vol. I, pp. 161-310, Academic Press, Inc., New York.
18. Schieberle, P., and Grosch, W. (1981) J. Am. Oil Chem. Soc. 58, 602-607.
19. Pryor, W.A., and Stanley, J.P. (1975) J. Org. Chem. 40, 3615-3617.
20. Pryor, W.A.; Stanley, J.P., and Blair, E. (1976) Lipids 11, 370-379.
21. Frankel, E.N.; Neff, W.E., and Bessler, T.R. (1979) Lipids 14, 961-967.
22. Neff, W.E., and Frankel, E.N. (1980) Lipids 15, 587-590.
23. Schöllner, R., and Herzschuh, R. (1966) Fette Seifen Anstrichm. 68, 469-475.
24. Schöllner, R., and Herzschuh, R. (1966) Fette Seifen Anstrichm. 68, 616-622.
25. Galliard, T.; Phillips, D.R., and Frost, D.J. (1973) Chem. Phys. Lipids 11, 173-180.
26. Schauenstein, E.; Esterbauer, H., and Zollner, H. (1977) Aldehydes in Biological Systems: Their Natural Occurrences and Biological Activities, Pion, London.
27. Kimoto, W.J., and Gaddis, A.M. (1974) J. Am. Oil Chem. Soc. 51, 307-311.
28. Grosch, W.; Schieberle, P., and Laskaway, G. (1981) in Flavour '81 (Schreier, P., ed.), pp. 443-448, Walter de Gruyter, Berlin.
29. Pryor, W.A. (1966) Free Radicals, McGraw-Hill, New York.
30. Denny, R.W., and Nickon, A. (1973) Org. React. 20, 133-336.
31. Frimer, A.A. (1979) Chem. Revs. 79, 359-387.
32. Barnard, D.; Hargrave, K.R., and Higgins, G.M.C. (1956) J. Chem. Soc. 2845-2849.

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METHODS

The Purification of Fatty Acid Methyl Esters By High Pressure Liquid Chromatography

EMANUELE BASCETTA, FRANK D. GUNSTONE and **CHARLES M. SCRIMGEOUR,**
Department of Chemistry, the University of St. Andrews, St. Andrews, Fife KY16 9ST, Scotland, United Kingdom

ABSTRACT

Procedures are described for the rapid purification of gram quantities of methyl oleate, methyl linoleate, methyl α- and γ-linolenates and methyl ricinoleate from appropriate natural oils by high pressure liquid chromatography (HPLC).
Lipids 19:801-803, 1984.

INTRODUCTION

The application of HPLC to lipid analysis has begun to gather momentum. Reversed-phase HPLC has been applied to triacylglycerols (1), fatty acids (2), methyl esters (3) and lecithins (4), and competes with gas liquid chromatography (GLC) in that both procedures separate lipids according to chain length and resolve *cis* and *trans* isomers to some extent (5). The latter resolution is enhanced if silver nitrate is dissolved in the eluting solvent (6), or impregnated in the stationary phase (7). Guerts Van Kessel et al (8) reported a HPLC procedure for the preparative purification of phospholipids. We now describe the use of preparative HPLC for the rapid purification of useful quantities of commonly required methyl esters.

MATERIALS AND METHODS

HPLC was carried out on a Waters Prep. LC/System 500 using refractive index detection. Flow rates were typically 200-250 ml/min. Columns (commercially available Prep. PAK-500 cartridges, 5.7 × 30 cm) were flushed with 1-2 l of solvent (methanol for reverse phase columns, 5% diethyl ether in petroleum ether for normal phase columns) prior to each run and then with the effluent to be used in the chromatographic separation until a steady base line was obtained. All solvents were of AR grade. Petroleum ether (b.p. 40-60 C) and diethyl ether were dried over calcium chloride and distilled before use. Methanol was refluxed over magnesium turnings for 2 hr before being distilled (twice).

Procedures for preparing methyl esters from fatty acids or directly from glycerides have been reported previously (9). Transesterified oils containing unwanted polar oxygenated material were purified by elution from a short column of silica (sorbsil) with petroleum ether.

Gas liquid chromatography was carried out on a Pye series 104 chromatograph equipped with flame ionization detector (FID) and fitted with a glass column (5′ × ¼″) packed with SP2300 (10%) as stationery phase on chromosorb WAW (100-120 mesh) operating at 200 C. Nitrogen was used as carrier gas at a flow rate of 55-65 ml/min.

RESULTS AND DISCUSSION

For the separations now described we selected solvents that were inexpensive, could be easily recovered for re-use, and still gave adequate separation in a reasonable time. Petroleum ether was generally used for normal phase chromatography and methanol for reverse phase systems. Our procedures, therefore, represent convenient methods for the rapid purification of useful quantities of commonly required methyl esters.

Methyl Oleate from Olive Oil Methyl Esters

Methyl oleate usually has been obtained from olive oil using one or more of urea crystallization, low-temperature crystallization and distillation as separation procedures (9).

Some years ago we recommended urea crystallization of olive acids to reduce the content of saturated acids below 1% followed by low temperature crystallization to raise the level of oleic acid to ⩾99% (9). The acids were then esterified and chromatographed to remove traces of colored impurities and oxidation products. We now find HPLC offers a convenient alternative.

It is generally advantageous to reduce the content of saturated esters by preliminary

urea fractionation (9), since palmitate forms a critical pair with some of the unsaturated C_{18} esters on a reverse phase column. Using olive esters in which the saturated ester content has been reduced to below 5% by urea fractionation, up to 15 g could be chromatographed in a single batch with recycling and pure methyl oleate (≥99%) obtained with 30 min of operator time. Figure 1 shows a typical HPLC trace for the isolation of methyl oleate from an upgraded sample of olive esters [7.5g: oleate (76.8%), linoleate (22.0%), palmitate (0.9%) and palmitoleate (0.3%)]. After 3 recycles, 2 peaks are observed with a resolution of 0.96 and the linoleate can be shaved off the major peak. Collection of the majority of the oleate peak on the subsequent pass then gives pure ester containing only traces of palmitate (0.6%) and palmitoleate (0.2%). With an aged column (i.e. one that has been used on several occasions previously) it may be necessary to recycle more than 3 times, though we found no further improvement after 6 recycles.

Methyl Linoleate from Maize, Sunflower, or Evening Primrose Oils

Low-temperature crystallization (10), urea fractionation (11,12), solvent partition of mercury complexes (13), liquid-liquid extraction (14), adsorption chromatography (15) and silver ion chromatography (16) have been used to isolate methyl linoleate (≥95% pure) from suitable seed oils. For example, Gunstone et al (9) isolated methyl linoleate (≥99% pure, 300-325 g) from Evening Primrose oil (1 Kg) containing 70-75% of linoleic acid by a combination of low-temperature crystallization, urea fractionation and column chromatography.

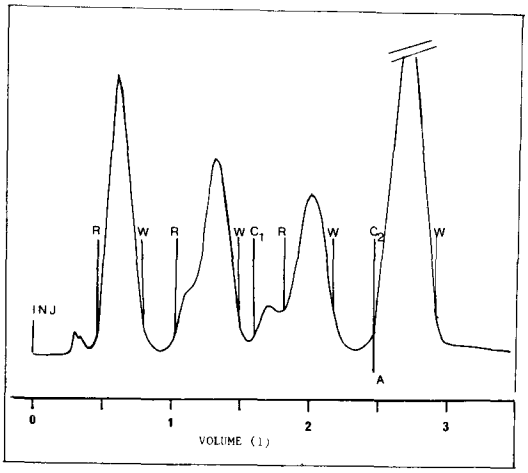

FIG. 1. Isolation of pure methyl oleate from upgraded olive esters. R = recycle, W = to waste, C = to collection, A = change in attenuation. Column Prep. PAK-500/C_{18} cartridge (5.7 × 30 cm), solvent methanol, flow rate 250 ml/min, load 7.5 g.

We now have isolated methyl linoleate from the esters derived from maize oil, sunflower oil or Evening Primrose oil on a reverse phase column using methanol as eluting solvent. All 3 sources proved to be equally satisfactory, and yields of methyl linoleate (≥99% pure) were in excess of 65% of the theoretical yield in each case. Recycling was necessary with shaving of the leading and trailing edges to remove linolenate and monoene/saturated esters respectively. In general, 6 recycles were needed taking 34 min and loads up to 13 g were routinely handled.

Without shaving, linoleate of about 90% purity can be obtained after recycling in about 17 min, but shaving is essential to get higher purity. Aged columns also gave pure esters but in slightly lower yields (50-55%).

Methyl α-Linolenate from Linseed Oil

The conventional techniques of low-temperature and urea crystallization do not readily furnish pure methyl α-linolenate because of the difficulty of separating linoleate and α-linolenate. Gunstone et al (9) previously recommended (a) crystallization of linseed acids (55-60% linolenic) from acetone at -75 C to raise the concentration of linolenic acid to 65-70% (b) urea crystallization to raise this further (90-92%), and (c) purification of methyl esters by repeated column chromatography on silica. The method produces about 120 g of pure α-linoleate (>99%) per kilogram of linseed oil at best and is very time consuming.

Reverse phase preparative HPLC of linseed esters (18:3 48.1%, 18:2 29.1%, 18:1 21.2%, and others 1.6%) gave a colorless product (about 96% pure) in 28 min. In a typical run, 8 recycles were performed with continuous shaving of the trailing edge and collecting the first 70% of the final recycle peak. Palmitate and palmitoleate were the major contaminants. A preliminary urea fractionation removes these C_{16} esters and α-linolenate (≥99%) is then obtained. About 7-8 g of linseed esters can be purified in a single run with a recovery of 1.4-1.7 g of pure α-linolenate.

Methyl γ-Linolenate from Evening Primrose Oil

Evening Primrose oil is the most convenient source of methyl γ-linolenate and can furnish methyl linoleate at the same time. Because of the low concentration of γ-linolenate (7-9%) in the oil, it is better to raise this to 50% or better by urea fractionation and low-temperature

crystallization. HPLC of an upgraded fraction (18:3 49%, 18:2 27%, 18:1 24%) gave γ-linolenate (>97% pure) after 4 recycles in 28 min. Batches of ester purified to this level and rechromatographed 8 g at a time gave pure γ-linolenate (≥99.5% pure) in 20 min.

As noted earlier (9), it is easier to separate γ-linolenate from linoleate than α-linolenate from linoleate. This also was evident in HPLC separation. After 4 recycles (without shaving), the resolution between γ-linolenate and linoleate was 0.78, whereas with linoleate/α-linolenate no resolution was apparent even after 8 recycles.

Methyl Ricinoleate from Castor Oil

Castor oil contains ricinoleic acid (ca. 90%) along with palmitate, stearate and unsaturated C_{18} acids. Methyl ricinoleate is easily isolated from castor esters by adsorption chromatography on columns or thin layers, the more polar hydroxy ester being more strongly adsorbed than the less polar impurities.

Methyl ricinoleate was purified easily by normal phase HPLC. Loads of up to 15 g can be tolerated, and pure ester is obtained in a single pass within 15 min. We achieved this satisfactorily with petroleum ether containing diethyl ether (4:1), but obtained better results with petroleum ether containing propan-2-ol (99:1). This has the added advantage that the petroleum ether can be recovered easily for re-use.

ACKNOWLEDGMENTS

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REFERENCES

1. El-Hamdy, A.H., and Perkins, E.G. (1981) J. Am. Oil Chem. Soc. 58, 49 and 867; Herslof, B.; Podlaha, O., and Toregard, B. (1979), J. Am. Oil Chem. Soc. 56, 864; Plattner, R.D. (1981) J. Am. Oil Chem. Soc. 58, 638; Plattner, R.D.; Spencer, G.F., and Kleiman, R. (1977) J. Am. Oil Chem. Soc. 54, 511; Peterssen, B.; Podlaha, O., and Toregard, B. (1981) J. Am. Oil Chem. Soc. 58, 1005.
2. Funk, M.O,; Isaac, R., and Porter, N.A. (1976) Lipids 11, 113.
3. Schofield, C.R. (1975), Anal. Chem. 47, 1417; Scholfield, C.R. (1975) J. Am. Oil Chem. Soc. 52, 36.
4. Carroll, R.M., and Rudel, L.L. (1981) J. Lipid Res. 22, 359.
5. Warthen, J.D. (1975) J. Am. Oil Chem. Soc. 52, 151.
6. Heath, R.R.; Tumlinson, J.D., and Doolittle, R.E. (1977) J. Chromatogr. Sci. 15, 10; Vonach, B., and Schomburg, G. (1978) J. Chromatogr. 149, 417.
7. Aitzetmuller, K.J. (1975) J. Chromatogr. Sci. 13, 454; Jones, A.D.; Smith, E.C., and Hammond, E.W. (1980) J. Chromatogr. 188, 205.
8. Guerts Van Kessel, W.S.M.; Tieman, M., and Demel, R.A. (1981) Lipids 16, 58.
9. Gunstone, F.D.; McLaughlan, J.; Scrimgeour, C.M., and Watson, A.P. (1976) J. Sci. Fd. Agric. 27, 675.
10. Sreenivasan, B.; Brown, J.B.; Jones, E.P.; Davison, V.L., and Nowakowska, J. (1962) J. Am. Oil Chem. Soc. 39, 255.
11. Johnson, A.R., and Ali, G.M. (1961) J. Am. Oil Chem. Soc. 38, 453.
12. Swern, D., and Parker, W.E. (1953) J. Am. Oil Chem. Soc. 30, 5.
13. Stearns, E.M.; White, H.B., and Quakenbush, F.W. (1962) J. Am. Oil Chem. Soc. 39, 61.
14. Beal, R.E., and Brekke, O.L. (1959) J. Am. Oil Chem. Soc. 36, 397.
15. Riemenschnieder, R.W.; Herb, S.F., and Nichols Jr., P.L. (1949) J. Am. Oil Chem. Soc., 26, 371.
16. Adlof, R.O., and Emken, E.A. (1980) J. Am. Oil Chem. Soc. 57, 276.

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The Thiobarbituric Acid Test for Lipid Peroxidation: Structure of the Adduct with Malondialdehyde

VASU NAIR and **GREGORY A. TURNER**, *Department of Chemistry, University of Iowa, Iowa City, Iowa 52242*

ABSTRACT

The complete structure of the red crystalline 2:1 adduct from thiobarbituric acid and malondialdehyde has been unambiguously determined by FTIR and high-field ^{1}H and ^{13}C NMR studies. *Lipids* 19:804-805, 1984.

INTRODUCTION

The thiobarbituric acid (TBA) is a commonly used method for the detection of peroxidation of unsaturated fatty acids and lipids (1,2). It is dependent on the development of a red pigment resulting from the reaction of TBA with oxidized lipids. It originally was suggested by Sinnhuber, et al. (3) that the red compound formed in the TBA test probably was a 2:1 adduct of TBA and malondialdehyde (MDA). However, several types of compounds other than MDA give positive TBA tests (4-7). Also, the presence of metal ions strongly influences the results (8).

Despite these limitations, the TBA test continues to be useful when used judiciously in studies of lipid peroxidation. Although the early proposed structural nature of the adduct of TBA and MDA is still cited, complete establishment of the structure of this red pigment is lacking. We now wish to report on a detailed and unambiguous assignment of the structure of this adduct.

EXPERIMENTAL PROCEDURES

The adduct of TBA and MDA was prepared and purified as described previously (3), except that pure sodium malondialdehyde (9) instead of 1,1,3,3-tetraethoxypropane was used in the preparation. The dark red needles that formed melted above 350 C. The high-field ^{1}H and ^{13}C NMR data, including delayed decoupling experiments, were determined on a Bruker WM-360 pulse Fourier transform instrument. The UV-visible spectra were recorded on a Varian-Cary Model 219 spectrophotometer. Fourier transform IR measurements were made on an IBM Model 98 instrument.

RESULTS AND DISCUSSION

The crystalline TBA-MDA adduct correctly analyzed for $C_{11}H_8N_4O_4S_2$. Its UV-visible spectrum in H_2O showed absorptions at λ_{max} 532 nm (ϵ = 159,200), 305 nm (ϵ = 11,250), and 243 nm (ϵ = 23,000), indicative of a highly conjugated system. The FTIR spectrum (KBr) was very diagnostic and exhibited bands characteristic of OH and NH stretching (broad peaks at 3490, 3200 cm^{-1}), amide carbonyl stretching (1633, 1670(sh) cm^{-1}), C-N vibration of S ‖ -C-NH- (1494 cm^{-1}), OH bending (1361 cm^{-1}), C-O stretching (1210, 1177 cm^{-1}), and thiomide C=S stretching (1127 cm^{-1}). The S-H stretching vibration which normally occurs at about 2500 cm^{-1} was not observed.

The high-field 360 MHz ^{1}H NMR spectrum of the adduct in DMSO-d_6 showed only 4 types of resonances. There were 5 exchangeable hydrogens, 3 appearing as a broad peak ($W_{1/2}$ = 41.7 Hz) at δ 6.11 and 2 appearing as a broad peak ($W_{1/2}$ = 10.8 Hz) at δ 11.52. The presence of vinyl protons of the MDA derived component easily could be discerned as a doublet (J = 13.9 Hz) integrating for 2 hydrogens and a triplet (J = 13.9 Hz) integrating for 1 hydrogen. No -SH resonance was observed (Table 1). The 90.56 MHz ^{13}C NMR data in DMSO-d_6 suggested that the adduct had considerable symmetry within its structure. The 11 carbons of the molecule showed only 5 resonances. Delayed decoupled high-field ^{13}C NMR experiments revealed the presence in the adduct of 3 different quaternary carbons and 2 types of tertiary carbons. A broadened peak at δ 161.9 was interpreted as being due to the presence of C=S of a thioamide. The carbons of the MDA moiety were assigned with the aid of the delayed-decoupling data to resonances at δ 117.5 and δ 157.4. The absorption at δ 176.3 was assigned as being due to the presence of amide carbons and that at δ 101.3 to the remaining two equivalent ring carbons (Table 2). The assignments are consistent with those expected on the basis of electronic, tautomeric

and multiplicity considerations, and also on the basis of comparisons with ^{13}C NMR spectra of some compounds with related moieties (10-12).

In conclusion, the combined spectroscopic data are totally consistent with two spectrally equivalent tautomeric structures **1** and **2**. It should be mentioned, however, that variation in concentration of solution and the presence of trace contaminants may cause prototropic shifts to favor equilibrating structures similar to **1** and **2** but bearing 3 hydroxyl and 2 amide hydrogens. Formation of the 2:1 adduct of TBA and MDA probably is initiated by nucleophilic attack involving carbon-5 of TBA onto carbon-1 of MDA followed by dehydration and similar subsequent reaction of the intermediate 1:1 adduct with a second molecule of TBA.

TABLE 1

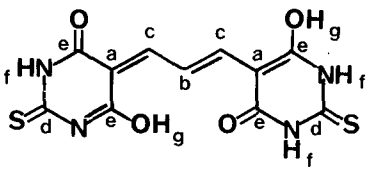
360 MHz ^1H NMR Data (in DMSO-d_6)

δ (ppm), TMS	Assignment
6.11 (s, br, 3H) (variable)	f
7.72 (d, J = 13.9 Hz, 2H)	c
8.56 (t, J = 13.9 Hz, 1H)	b
11.52 (s, br, 2H)	g

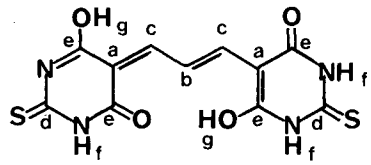
TABLE 2

90.56 MHz ^{13}C NMR Data (in DMSO-d_6)

δ (ppm), TMS	Delayed Decoupling	Assignment
101.3	C	a
117.5	CH	b
157.4	CH	c
161.9	C	d
176.3	C	e



STRUCTURE 1



STRUCTURE 2

ACKNOWLEDGMENTS

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REFERENCES

1. (1980) Autoxidation in Food and Biological Systems (Simic, M.G. and Karel, M., eds.) Plenum Press, New York.
2. Gray, J.I. (1978) J. Am. Oil Chem. Soc. 55, 539-546.
3. Sinnhuber, R.O.; Yu, T.C., and Yu, Te Chang (1958) Food Res. 23, 626-633.
4. Porter, N.A.; Nixon, J., and Issac, R. (1976) Biochim. Biophys. Acta 441, 506-512.
5. Pryor, W.A. (1980) in Molecular Basis of Environmental Toxicity (Bhatnagar, R.S., ed.) pp. 15-20, Ann Arbor Science, Ann Arbor.
6. Baumgartner, W.A.; Baker, N.; Hill, V.A., and Wright, E.T. (1975) Lipids 10, 309-311.
7. Marcuse, R., and Johansson, L. (1973) J. Am. Oil Chem. Soc. 50, 387-391.
8. Willis, E.D. (1964) Biochim. Biophys. Acta 84,475-477.
9. Nair, V.; Vietti, D.E., and Cooper, C.S. (1981) J. Am. Chem. Soc. 103, 3030-3036.
10. Levy, G.C.; Lichter, R.L., and Nelson, G.L. (1980) Carbon-13 Nuclear Magnetic Resonance Spectroscopy, Wiley-Interscience, New York.
11. Rosenthal, S.N., and Fendler, J.H. (1976) in Advances in Physical Organic Chemistry (Gold, V. and Bethel, D., eds.) pp. 279-424, Academic Press, New York.
12. Johnson, L.F., and Jankowski, W.C. (1972) Carbon-13 NMR Spectra, Wiley, New York.

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COMMUNICATIONS

Essential Fatty Acids in Plasma, Red Blood Cells and Liver Phospholipids in Common Laboratory Animals as Compared to Humans

DAVID F. HORROBIN*, Y-S. HUANG, S.C. CUNNANE and **M.S. MANKU**, *Efamol Research Institute, P.O. Box 818, Kentville, Nova Scotia B4N 4H8, Canada*

ABSTRACT

Essential fatty acids were estimated in plasma, red blood cells and liver total phospholipids in rats, mice, hamsters, guinea pigs, rabbits and humans. There were large species differences, suggesting that different species levels should be borne in mind when choosing an animal for a particular study. The 2 species most susceptible to atheroma, the guinea pig and the rabbit, had very high levels of linoleic acid and low levels of linoleic acid metabolites. n-3 fatty acid levels were low in guinea pigs and rabbits and the ratio of n-3 to n-6 fatty acids also was low in the rat. Mice and hamsters had the highest n-3 levels, suggesting they may be the best species to use for studies on the roles of n-3 essential fatty acids. Mice and hamsters and, in some respect rats, were closest to humans in their fatty acid patterns. *Lipids* 19:806-811, 1984.

INTRODUCTION

While studies on essential fatty acid deficiency have been carried out in most laboratory animals, the great majority have been performed in the rat. Comparisons of liver and brain essential fatty acid (EFA) levels have been made in larger animals (1), but surprisingly there does not appear to be in the literature any survey of EFA levels, using the same methodology, in the commonly used laboratory animals. Much information is available about EFA levels in rats, but there are no comparative studies. We have, therefore, measured EFA levels in plasma, red cell membranes, and liver total phospholipids in rats, mice, hamsters, guinea pigs, rabbits and humans. The results show considerable differences among species, which should be borne in mind when choosing a species for a particular study.

MATERIALS AND METHODS

All samples were from young adult males unless otherwise stated. All animals were being fed standard laboratory chows. The rats, mice and hamsters were fed on Purina rat chow and the rabbits and guinea pigs on Purina guinea pig chow. The lipid compositions of these chows are shown in Table 1. As can be seen, they are virtually identical. Animals were killed by exsanguination under ether anesthesia following an overnight fast during which the animals had access to water only. Blood was collected via the abdominal aorta into a test tube containing EDTA (1 mg/ml blood). Plasma was separated from erythrocytes by centrifugation. Livers were excised, rinsed in ice cold saline and frozen for lipid extraction.

Plasma and liver lipids were extracted by the method of Folch, et al (2) and erythrocyte lipids by that of Dodge and Phillips (3). The phospholipid fractions were separated by thin layer chromatography (TLC), methylated and subjected to gas-liquid chromatography (GLC) for determination of fatty acid composition as previously described (4,5). A 10% Silar 10 C on Gas Chrom Q column was used with a

TABLE 1

Fatty Acid Composition of Diets

Fatty Acid	Rat Chow 5001	Guinea Pig Chow 5025
14:0	1.5	1.1
14:1	–	0.5
16:0	21.9	20.1
16:1	3.1	3.7
18:0	9.9	8.7
18:1	32.2	30.4
18:2n-6	24.3	27.1
18:3n-3	3.7	3.3
Others	3.4	5.1

The rat chow was used for the mice and hamsters also.

The guinea pig chow was also used for rabbits.

Results are shown as mg/100 mg total fatty acids present.

Total fat in the rat chow was 4.5 g/100 g and in the rabbit chow 4 g/100 g.

*To whom correspondence should be addressed.

Hewlett Packard 5880A machine with automated integration.

The following animals were used: 7 Sprague-Dawley rats; 9 Swiss albino mice; 8 Syrian Golden Hamsters; 8 Hartley guinea pigs; 3 New Zealand White rabbits.The human plasma and red cell samples were from normal university students as previously described (5). There were 32 males and 18 females, but because no sex differences were observed they were grouped together. Samples were taken in the morning after an overnight fast. Caution must be expressed concerning the human liver samples because they were not from normal individuals: they were taken from nine women and 1 man undergoing by-pass surgery for refractory obesity.

RESULTS

The results are shown in Tables 2-4. In the animals many of the fatty acids showed little variation among individuals, leading to very small standard deviations, and emphasizing the importance of the striking differences among species. The details can be obtained by inspection of the tables, but the following points are worth emphasizing:

1. Linoleic acid levels were exceptionally high in all 3 tissues in the rabbit and the guinea pig.

2. Arachidonic acid levels were exceptionally high in the rat.

3. Total metabolites of 18:2n-6 were high in the rat with the human consistently second. Levels of arachidonic acid were substantial in humans, but this metabolite was not so dominant as in the rat. 18:2n-6 metabolites were consistently low in all tissues in the rabbit and in the liver and plasma in guinea pigs. The ratio of 18:2n-6 to its metabolites was consistently highest in the guinea pig and rabbit and lowest in the rat and human.

4. n-6 EFAs with 22 carbons were much more evident in plasma and red cells in humans than in other species.

5. 18:3n-3 was low in all fractions in all species. Concentrations of 18:3n-3 metabolites were exceptionally low in guinea pigs and rabbits and consistently rather high in mice and hamsters.

6. The ratio of 18:2n-6 metabolites to 18:3n-3 metabolites was high in rats, guinea pigs and rabbits. This ratio was lowest in mice and hamsters, with humans intermediate.

DISCUSSION

This survey provides background information which can be used when choosing an appropriate species for a particular study. It is commonly believed that fatty acid patterns are closely related to diet. While this may be true of other lipid components such as triglycerides, cholesterol esters and free fatty acids, it does not appear to be true of the phospholipids which were measured in this study. For all 4 animals the lipid composition of the diet was virtually identical, yet the fatty acid compositions of plasma, red cell and liver phospholipids showed striking differences. It was not possible to put our normal human controls on a standard diet, but at least as far as phospholipids are concerned there seems to be considerable consistency in different populations. Plasma phospholipid fatty acid compositions were recently found to be virtually identical in populations from Minnesota and Nova Scotia living about 2000 miles apart (5,6).

Some general conclusions can be drawn from this study:

1. Each species has a characteristic phospholipid fatty acid composition which cannot be explained by differences in dietary lipid intake and must, therefore, be related to species differences in essential fatty acid metabolism.

2. The 2 species of animals most susceptible to atheroma, the guinea pig and the rabbit, have by far the highest levels of linoleic acid. Although linoleic acid rich diets may protect against cardiovascular diseases in humans (7), it is unlikely that linoleic acid itself is the agent responsible. Swell, et al (8) showed that the susceptibility of a species to atherosclerosis was inversely related to the level of arachidonic acid in plasma cholesterol esters. The results of this survey are consistent with the Swell study and also with the observation that a linoleic acid metabolite, rather than linoleic acid itself, seems to be important in lowering cholesterol levels in humans (9).

3. The exceptionally high levels of arachidonate in the rat make it a good species for studying arachidonate metabolism. On the other hand, results obtained in the rat may, for this same reason, have only limited application to other species, including humans.

4. Amounts of 18:3n-3 metabolites, especially when expressed in relation to 18:2n-6 metabolites, are low in rabbits, guinea pigs and rats. These species appear to be able to function with only small amounts of n-3 EFAs and so may be inappropriate for studies on the effects of n-3 deficiencies. The hamster or the mouse would appear to be the species of choice.

In conclusion, species variations in EFA levels suggest that the laboratory animal to be used in an EFA study must be chosen with care.

TABLE 2

Fatty Acids in Total Plasma Phospholipids in Various Species

	Human	Rat	Mouse	Hamster	Guinea Pig	Rabbit
Non-EFAs						
16:0	23.90 ± 7.20	15.1 ± 2.5	26.6 ± 0.5	23.7 ± 1.1	12.3 ± 1.2	22.1 ± 0.9
18:0	11.61 ± 1.32	16.3 ± 4.9	13.2 ± 0.1	13.7 ± 3.9	25.7 ± 1.6	14.1 ± 1.8
18:1n-9	13.50 ± 2.20	6.6 ± 1.0	8.8 ± 0.7	10.0 ± 0.3	12.1 ± 0.7	8.7 ± 0.5
n-6 EFAs						
18:2	21.45 ± 2.60	18.40 ± 2.0	26.1 ± 1.5	30.0 ± 1.8	38.0 ± 1.9	43.8 ± 1.8
18:3	0.16 ± 0.08	—	0.5 ± 0.1	—	—	—
20:3	3.06 ± 0.60	0.9 ± 0.1	2.3 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	tr
20:4	11.36 ± 1.67	33.6 ± 3.4	9.0 ± 0.6	7.8 ± 0.9	7.0 ± 0.3	9.1 ± 0.4
22:4	0.73 ± 0.26	—	—	—	0.7 ± 0.3	—
22:5	1.12 ± 0.67	—	—	—	0.8 ± 0.3	—
18:2 metabolites	16.27	34.5	11.8	8.5	9.3	9.1
18:2/metabolites	1.23	0.53	2.21	3.53	4.09	4.81
C22 acids	1.85	—	—	—	1.5	—
n-3 EFAs						
18:3	—	tr	0.3 ± 0.02	—	1.2 ± 0.4	—
20:5	1.01 ± 0.36	0.5 ± 0.1	0.9 ± 0.1	2.7 ± 0.5	—	—
22:5	0.93 ± 0.27	0.8 ± 0.2	—	0.9 ± 0.1	0.6 ± 0.2	—
22:6	3.54 ± 0.89	6.7 ± 0.9	9.8 ± 0.3	8.3 ± 1.3	0.7 ± 0.2	—
18:3 metabolites	5.48	8.0	10.7	11.9	1.3	—
18:2n-6 metabolites/ 18:3n-6 metabolites	2.97	4.31	1.1	0.71	7.15	—

Results are expressed in mg/100 mg total fatty acids present. Each figure represents the mean ± SD.

TABLE 3

Fatty Acids in Total Red Cell Phospholipids in Various Species

	Human	Rat	Mouse	Hamster	Guinea Pig	Rabbit
Non-EFAs						
16:0	20.68 ± 1.85	22.1 ± 0.6	27.3 ± 0.8	19.4 ± 0.6	12.0 ± 0.6	20.0 ± 0.3
18:0	14.71 ± 1.56	14.8 ± 3.6	10.5 ± 0.6	12.6 ± 1.5	24.9 ± 3.4	16.8 ± 2.4
18:1n-9	14.83 ± 1.70	8.9 ± 0.6	14.2 ± 3.3	15.8 ± 0.3	9.9 ± 0.4	12.3 ± 0.5
n-6 EFAs						
18:2	9.78 ± 1.70	11.4 ± 1.2	14.0 ± 1.0	17.6 ± 0.8	19.9 ± 3.3	37.6 ± 1.3
18:3	—	—	—	—	—	—
20:3	1.37 ± 0.36	0.8 ± 0.1	1.5 ± 0.04	0.7 ± 0.1	1.3 ± 0.3	0.7 ± 0.1
20:4	15.13 ± 2.06	30.0 ± 2.8	14.3 ± 0.3	16.6 ± 0.6	18.0 ± 2.3	6.9 ± 0.5
22:4	5.54 ± 1.42	0.8 ± 0.1	1.1 ± 0.07	1.8 ± 0.3	—	1.0 ± 0.5
22:5	3.99 ± 1.07	0.3 ± 0.06	1.3 ± 0.02	2.7 ± 0.3	2.0 ± 0.3	—
18:2 metabolites	26.03	31.9	18.2	21.8	21.3	8.6
18:2/metabolites	0.38	0.36	0.77	0.81	0.93	4.37
C22 acids	9.53	1.1	2.4	4.5	2.0	1.0
n-3 EFAs						
18:3	—	—	0.6 ± 0.05	0.5 ± 0.03	1.2 ± 0.2	2.2 ± 0.1
20:5	0.65 ± 0.21	0.8 ± 0.3	1.6 ± 0.1	0.7 ± 0.1	—	0.2 ± 0.02
22:5	2.53 ± 0.92	0.3 ± 0.06	1.3 ± 0.02	2.7 ± 1.2	1.8 ± 0.3	1.1 ± 0.4
22:6	4.20 ± 1.55	5.2 ± 0.5	11.5 ± 0.5	3.9 ± 0.8	0.6 ± 0.2	—
18:3 metabolites	7.38	6.3	14.4	7.3	2.4	1.3
18:2n-6 metabolites/ 18:3n-6 metabolites	3.53	5.06	1.26	2.99	8.88	6.61

Results are expressed in mg/100 mg total fatty acids present. Each figure represents the mean ± SD.

TABLE 4

Fatty Acid Concentrations in mg/100 mg in Total Liver Phospholipids

	Human	Rat	Mouse	Hamster	Guinea Pig	Rabbit
Non-EFAs						
16:0	22.3 ± 2.0	15.29 ± 1.01	23.63 ± 2.27	17.80 ± 0.89	11.4 ± 1.1	17.0 ± 1.4
18:0	15.9 ± 2.3	23.51 ± 0.69	10.93 ± 4.08	17.40 ± 1.67	27.7 ± 2.1	20.3 ± 1.9
18:1n-9	16.0 ± 1.7	6.22 ± 0.56	9.12 ± 0.38	8.95 ± 0.54	10.1 ± 0.7	8.2 ± 0.7
n-6 EFAs						
18:2	17.3 ± 2.3	13.53 ± 0.87	16.18 ± 2.37	18.3 ± 0.64	35.7 ± 1.9	36.5 ± 1.4
18:3	0.41 ± 0.13	—	0.10 ± 0.01	—	—	—
20:3	2.41 ± 0.51	1.09 ± 0.27	1.78 ± 0.07	0.90 ± 0.12	1.0 ± 0.1	1.5 ± 0.1
20:4	13.29 ± 1.88	29.41 ± 1.24	15.98 ± 2.55	13.1 ± 0.34	6.7 ± 0.6	10.1 ± 1.0
22:4	0.49 ± 0.11	0.31 ± 0.02	0.18 ± 0.01	0.25 ± 0.02	0.6 ± 0.1	0.5 ± 0.06
22:5	0.49 ± 0.11	0.10 ± 0.02	0.34 ± 0.23	0.41 ± 0.09	0.6 ± 0.1	0.6 ± 0.04
18:2 metabolites	17.09	30.91	18.38	14.55	8.9	12.7
18:2/metabolites	1.01	0.44	0.89	1.26	4.01	2.87
C22 acids	0.98	0.41	0.52	0.65	1.2	1.1
n-3 EFAs						
18:3	0.40 ± 0.13	0.32 ± 0.08	0.40 ± 0.01	0.24 ± 0.03	—	1.5 ± 0.1
20:5	0.74 ± 0.24	0.54 ± 0.15	1.26 ± 0.49	1.53 ± 0.48	0.1 ± 0.02	0.2 ± 0.04
22:5	0.83 ± 0.17	1.04 ± 0.21	0.95 ± 0.06	1.10 ± 0.20	0.4 ± 0.06	0.6 ± 0.04
22:6	6.94 ± 1.20	8.10 ± 0.53	17.80 ± 3.12	16.20 ± 0.62	0.6 ± 0.1	1.0 ± 0.1
18:3 metabolites	8.51	9.68	20.01	18.83	1.1	1.8
18:2n-6 metabolites/ 18:3n-6 metabolites	1.96	3.15	0.91	0.77	8.09	7.06

The human samples in this case only were from abnormal individuals.

REFERENCES

1. Crawford, M.S.; Casperd, N.M., and Sinclair, A.J. (1976) Comp. Biochem. Physiol. 54B, 395-401.
2. Folch, J.; Lees, M., and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497-509.
3. Dodge, J.T., and Phillips, G.B. (1967) J. Lipid Res. 8, 667-675.
4. Manku, M.S.; Horrobin, D.F.; Morse, N.; Kyte, V., and Jenkins, K. (1982) Prostaglandins Leukotr. Med. 9, 615-628.
5. Manku, M.S.; Horrobin, D.F.; Huang, Y-S., and Morse, N. (1983) Lipids, 18, 906-908.
6. Holman, R.T.; Smythe, L., and Johnson, S. (1979) Am. J. Clin. Nutr. 32, 2390-2399.
7. Grundy, S.M.; Bilheimer, D.; Blackburn, H.; Brown, W.V.; Kwiterovich, P.O.; Mattson, F.; Schonfeld, G., and Weidman, W.H. (1982) Circulation 65, 839A-854A.
8. Swell, L.; Field, H., and Treadwell, C.R. (1960) Proc. Soc. Exp. Biol. Med. 104, 325-328.
9. Horrobin, D.F., and Manku, M.S. (1983) Lipids 18, 558-562.

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Influence of Cholesterol Feeding and Dietary Restriction on Cholesterol Absorption in Rabbits

G. CORRAZE, C. LACOMBE* and M. NIBBELINK, *ERA 412 CNRS, Institute de Physiologie, rue Francois Magendie F-31400 Toulouse, France*

ABSTRACT

Plasma cholesterol and cholesterol absorption were measured in rabbits given either a standard or cholesterol-rich diet which were fed either ad libitum or reduced to 50% of the control ration. The results confirmed the aggravating effect of dietary restriction on the plasma cholesterol response to cholesterol feeding. Hypercholesterolemia was doubled when cholesterol feeding was associated with reduced dietary intake. The percentage of cholesterol absorbed increased significantly in cholesterol-fed rabbits on normal caloric ration, while dietary restriction had no effect on this parameter either with the standard or the cholesterol-rich diet. These data indicate that the mechanisms by which plasma cholesterol increases in response to cholesterol feeding involve increased cholesterol absorption. Nevertheless the aggravating effect of dietary restriction cannot be attributed to increased cholesterol absorption.

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INTRODUCTION

Among the various animal species, the rabbit is well known for its propensity to develop severe hypercholesterolemia when dietary cholesterol is increased (1-3). In addition, our previous results showed that the hypercholesterolemia could be markedly aggravated when cholesterol feeding is associated with restriction in dietary intake (4). The present study was designed to test the possible changes in intestinal cholesterol absorption.

MATERIALS AND METHODS

The experiment was carried out with four groups of 8 male New Zealand rabbits in the weight range of 2.4-2.8 kg. A standard rabbits' diet provided from UAR (Villemoisson/Orge) and the same diet supplemented with cholesterol were used. The rabbits were given daily the following dietary ration as previously described (4): Control (C), 160 g of standard diet; Restricted Control (RC), 80 g of standard diet; Cholesterol (Ch), 160 g of standard diet + 0.2 g/kg body weight of cholesterol; Restricted Cholesterol (R Ch), 80 g of standard diet + 0.2 g/kg body weight of cholesterol. During the course of the experiment, body weight and plasma cholesterol (5) were measured weekly.

After four weeks on the respective diets the intestinal absorption of cholesterol was measured with the dual isotope method of Zilversmit (6). The intravenous dose of ^{14}C-cholesterol (2.61 μCi/rabbit) and the oral dose of ^{3}H-cholesterol (8.28 μCi/rabbit) were administered simultaneously in the morning following an 18-hour fast. Plasma samples were then collected after 24, 48 and 96 hours. Isotope measurements were carried out after combustion of the plasma samples in an Oxymat apparatus (Inter-Technique). The samples were counted in Carbomax (Kontron Analytic) and in dioxane/toluene (7V/3V) 2% naphtalene, 0.7% butyl PBD for ^{14}C and ^{3}H respectively. The results are the mean of the three samples collected, as no change with time was observed in the values obtained.

RESULTS

Changes in body weight and in plasma cholesterol were as expected. Rabbits on normal caloric ration (Control and Cholesterol) continued their growth (229 ± 20 g of body weight increase), while rabbits on restricted diets (Restricted Control and Restricted Cholesterol) lost 164 ± 19 g during the course of the experiment. The increase in plasma cholesterol was doubled when cholesterol feeding was associated with dietary restriction, while no marked changes occurred in the Restricted Control group (Fig. 1).

The dietary intake and the intestinal cholesterol absorption data are presented in Table 1. The amount of cholesterol provided was adjusted to body weight (0.2 g/kg); therefore, the mean daily intake was less in the Restricted Cholesterol than in the Cholesterol group. On the other hand, the proportion in the diet was less in the Cholesterol group. Dietary restriction had no effect on the percentage of cholesterol absorbed either with the standard or the cho-

*To whom correspondence should be addressed.

lesterol rich diet. Conversely, cholesterol feeding without food restriction induced a significant increase in this parameter. Therefore, the total daily amount of cholesterol absorbed was consistently reduced in the Restricted Cholesterol compared with the Cholesterol group when expressed per animal as well as per kg body weight.

DISCUSSION

The homeostasis of body cholesterol under conditions of a high cholesterol intake depends on several mechanisms involving inhibition of endogenous synthesis, limitation of intestinal absorption and increased fecal excretion (7-9). In cholesterol-fed rabbits the marked expansion of the miscible pool of cholesterol shows a deficiency in these regulating systems (9). Rabbits in particular are known for their inability to compensate increased input of exogenous cholesterol by raising the excretion of neutral sterols and bile acids (10). Moreover, the present study shows that in rabbits on a cholesterol-rich diet the intestinal absorption of cholesterol is not limited but on the contrary is done of a more efficient rate.

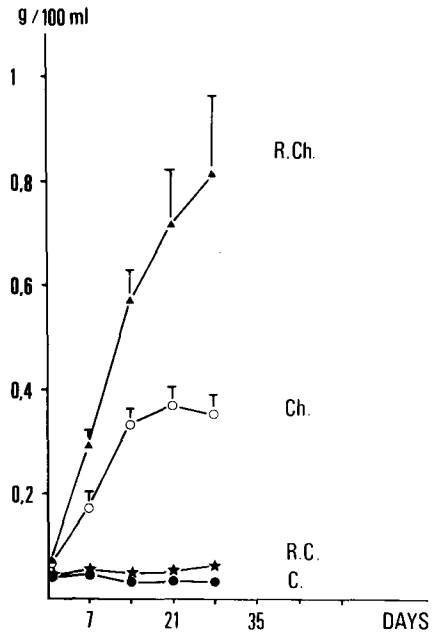

FIG. 1. Influence of dietary restriction on cholesterolemia in rabbits fed either standard or cholesterol-rich diets. C = Control; RC = Restricted Control; Ch = Cholesterol; R Ch = Restricted Cholesterol.

The rate of intestinal uptake of a micellar solution of cholesterol examined by an in vitro technique was seen to be reduced in mucosa originating from cholesterol-fed rabbits (11). Our results demonstrate that in vivo the overall result of the various steps in the whole process of cholesterol absorption is different. Present knowledge on the major rate-limiting steps was recently reviewed by Grundy (12). They are: the solubilization of cholesterol in mixed micelles which requires sufficient concentration of bile acids and other amphipathic substances; the movement of cholesterol through the unstirred layer of water adjacent to the surface of the luminal cells and, finally, the capacity for esterification by mucosal cells (12). Further study would be necessary to determine the mechanism by which cholesterol absorption is increased in cholesterol-fed rabbits. However, the discrepancy between our data and that of studies with isolated mucosa suggests the preponderance of the solubilization process.

In addition, the present results confirm the previous observation that limited feeding enhances the effect of increased dietary cholesterol on the rise in plasma cholesterol (4).

TABLE 1

Influence of Dietary Restriction on Cholesterol Absorption in Rabbits Fed Either Standard or Cholesterol-Rich Diets

	Dietary Cholesterol		Cholesterol Absorbed		
	mg/day	mg/100 g	Percent of dietary intake	mg/day/rabbit	mg/day/kg body weight
C	0	0	49.4 ± 3.5[a]*	0	0
RC	0	0	53.3 ± 2.8[a]*	0	0
Ch	556 ± 10.6[a]	348 ± 6.8[a]	68.2 ± 2.3[b]	379 ± 7.2[a]	136 ± 4.6[a]
R Ch	507 ± 9.7[b]	633 ± 12.1[b]	41.9 ± 7.3[a]	212 ± 4.1[b]	83.8 ± 14.6[b]

C = Control; RC = Restricted Control; Ch = Cholesterol; R Ch = Restricted Cholesterol. Values are means ± SEM of eight rabbits, [a,b]: superscript letters, indicate intergroups statistical difference means not sharing a common letter are significantly different ($P < 0.05$).

*Per cent of dietary intake presented for C and RC rabbits represents the ability of animals without cholesterol in their diet to absorb a single dose of orally administered cholesterol.

The measurement of cholesterol turnover carried out in the previous study showed an increased production rate of cholesterol in the Restricted Cholesterol compared to the Cholesterol group. Conversely, this parameter was reduced in food restricted rabbits on a standard diet. The fact that dietary restriction has an opposite effect on cholesterol turn-over with the cholesterol-rich or the standard diet suggested a difference in the intestinal absorption of cholesterol. In fact the present data show that the intestinal absorption of dietary cholesterol is not enhanced in Restricted Cholesterol rabbits but, conversely, the daily amount absorbed is reduced.

In our experimental conditions the amount of cholesterol ingested daily was adjusted to body weight (0.2 g/kg). Therefore, the proportion of cholesterol in the dietary ration was less in the Cholesterol than in the Restricted Cholesterol diet. Thus it can be hypothesized that more fatty acids would be available for the formation of mixed micelles with higher dietary intake providing more cholesterol in a suitable form for uptake by the mucosal cells to explain the increased cholesterol absorption in cholesterol-fed rabbits on normal caloric ration.

In conclusion, the present report showing the very efficient rate of cholesterol absorption in cholesterol-fed rabbits provides additional data to explain the severe hypercholesterolemia observed in these animals. On the other hand, the daily amount of cholesterol absorbed is consistantly less in food restricted rabbits. Therefore, the aggravating effect of dietary restriction when associated with cholesterol feeding cannot be attributed to increased cholesterol absorption. Further experiments, especially concerning the endogenous synthesis of cholesterol, are necessary to investigate the origin of the additional increase in plasma cholesterol which occurs with restriction in dietary intake.

REFERENCES

1. Camejo, G,; Bosch, V.; Arreaza, C., and De Mendez, H. (1973) J. Lipid Res., 14, 61-68.
2. Shore, B. and Shore, V. (1976) Adv. Exp. Med. Biol., 67, 123-141.
3. Ross, A.C.; Minick, C.R., and Zilversmit, D.B. (1978) Atherosclerosis, 29, 301-315.
4. Lacombe, C.; Corraze, G., and Nibbelink, M. (1983) Lipids, 18, 306-312.
5. Roschlau, P,; Bernt, E., and Gruber, W. (1974) Z. Klin. Chem. Klin. Biochem., 12, 403-407.
6. Zilversmit, D.B. (1972) Proc. Soc. Exp. Biol. Med., 140, 862-865.
7. Dietschy, J.M. and Wilson, J.D. (1970) N. Engl. J. Med., 282, 1128-1138.
8. Dietschy, J.M. and Wilson, J.D. (1970) N. Engl. J. Med., 282, 1179-1183.
9. Dietschy, J.M. and Wilson, J.D. (1970) N. Engl. J. Med., 282, 1241-1249.
10. Hellström, K. (1965) Acta Physiol. Scand., 63, 21-35.
11. Thomson, A.B.R. (1982) Am. J. Clin. Nutr., 35, 556-565.
12. Grundy, S.M. (1982) Ann. Rev. Nutr., 3, 71-96.

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ERRATUM

In the September 1984 issue of *Lipids,* one technical paper was omitted from the Table of Contents. "Occurrence of a 22:2 Nonmethylene Interrupted Dienoic Fatty Acid and Its Seasonal Distribution Among Lipids and Tissues of the Fresh Water Bivalve *Diplodon delodontus* From an Isolated Environment" appeared on pages 649 through 655. It was written by C. E. Irazu, R. J. Pollero and R. R. Brenner.

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In conclusion, the present report showing the very efficient rate of cholesterol absorption in cholesterol-fed rabbits provides additional data to explain the severe hypercholesterolemia observed in these animals. On the other hand, the daily amount of cholesterol absorbed is consistantly less in food restricted rabbits. Therefore, the aggravating effect of dietary restriction when associated with cholesterol feeding cannot be attributed to increased cholesterol absorption. Further experiments, especially concerning the endogenous synthesis of cholesterol, are necessary to investigate the origin of the additional increase in plasma cholesterol which occurs with restriction in dietary intake.

REFERENCES

1. Camejo, G,; Bosch, V.; Arreaza, C., and De Mendez, H. (1973) J. Lipid Res., 14, 61-68.
2. Shore, B. and Shore, V. (1976) Adv. Exp. Med. Biol., 67, 123-141.
3. Ross, A.C.; Minick, C.R., and Zilversmit, D.B. (1978) Atherosclerosis, 29, 301-315.
4. Lacombe, C.; Corraze, G., and Nibbelink, M. (1983) Lipids, 18, 306-312.
5. Roschlau, P,; Bernt, E., and Gruber, W. (1974) Z. Klin. Chem. Klin. Biochem., 12, 403-407.
6. Zilversmit, D.B. (1972) Proc. Soc. Exp. Biol. Med., 140, 862-865.
7. Dietschy, J.M. and Wilson, J.D. (1970) N. Engl. J. Med., 282, 1128-1138.
8. Dietschy, J.M. and Wilson, J.D. (1970) N. Engl. J. Med., 282, 1179-1183.
9. Dietschy, J.M. and Wilson, J.D. (1970) N. Engl. J. Med., 282, 1241-1249.
10. Hellström, K. (1965) Acta Physiol. Scand., 63, 21-35.
11. Thomson, A.B.R. (1982) Am. J. Clin. Nutr., 35, 556-565.
12. Grundy, S.M. (1982) Ann. Rev. Nutr., 3, 71-96.

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ERRATUM

In the September 1984 issue of *Lipids,* one technical paper was omitted from the Table of Contents. "Occurrence of a 22:2 Nonmethylene Interrupted Dienoic Fatty Acid and Its Seasonal Distribution Among Lipids and Tissues of the Fresh Water Bivalve *Diplodon delodontus* From an Isolated Environment" appeared on pages 649 through 655. It was written by C. E. Irazu, R. J. Pollero and R. R. Brenner.

Polyunsaturated Fatty Acids and Neutral Lipids in Developing Larvae of the Oyster, *Crassostrea virginica*[1]

FU-LIN E. CHU* and **KENNETH L. WEBB**, *School of Marine Science and Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062*

ABSTRACT

The fatty acid composition of oyster larvae at various stages, as well as of the algal diet, were determined by gas liquid chromatography (GC). Saturated fatty acids are the major fatty acid components in all larval stages and account for 34-62%, 30-35% and 35-81% of the neutral, polar and total lipids of algal-fed larvae respectively. Weight percentage of saturated fatty acid in "starved" larvae was consistently higher (63-81%) during the whole period. The total polyunsaturated fatty acids were higher in the polar lipids than in the neutral lipids. The concentration of the ω3 fatty acids also was comparatively higher in the polar lipids than in the neutral lipids. In the total and neutral lipid fractions, the weight percentage of polyunsaturated and ω3 fatty acids was higher in the eyed than in the pre-eyed (pediveliger) larvae. Eicosapentaenoic acid (20:5ω3) and 22:6ω3 were not detected in lipids of "starved" and young larvae. There was an accumulation of 20:5ω3, 22:6ω3, and total ω3 fatty acids in the older larvae. Lipid classes were separated by thin layer chromatography (TLC). There was no qualitative change in lipid composition during larval development, but a marked increased of triacylglycerol in larvae up to the stage of maturation in algae-fed larvae.
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INTRODUCTION

Previous studies (1-6) have demonstrated the importance of lipids and fatty acids in oyster larval growth and development. Creekman (3) reported that the lipid content of the egg and that of the resulting larvae of American oyster *(Crassostrea virginica)* were correlated and that a greater egg lipid content significantly increased larval growth, vigor, set and successful metamorphosis. Recent studies by Gallager and Mann (6) on the same subject with 2 bivalve species, *Mercenaria mercenaria* and *C. virginica*, have produced results which agreed with those of Creekman. Similarly, Helm et al. (7) have reported that the viability of *Ostrea edulis* larvae was related to their lipid content, particularly the neutral lipid, at the time of liberation. Holland and Spencer (2) indicated that accumulation of neutral lipid (from 8.8 to 23.2%) occurred during development of *O. edulis* larvae. The larval growth rate of *O. edulis* and *C. virginica* was found to be unrelated to the quantity of total lipid (4,5), but closely related to the triacylglycerol content present in the algal diet (4). These results support the conclusion that lipid supplies most of the energy requirements of larvae during periods of both growth and starvation (1,8). However, all of these studies were concerned only with the changes of the total lipid or neutral lipid during larval development. In the literature there is only one paper (4) that deals with the determination of fatty acid composition of *C. gigas* larvae, and that study involved only one stage (6-day old larvae) of larval development. The objectives of the experiments reported here were twofold: to examine the changes in fatty acid and neutral (in terms of triacylglycerol) lipid of oyster larvae during development, and to assess the nutritional significance of these changes in larval development.

*To whom correspondence should be addressed.

[1]Contribution number 1195 of the Virginia Institute of Marine Science, Gloucester Point, VA 23062

MATERIALS AND METHODS

Growth Experiments

The larval growth experiment was performed twice. Conditioned adult oysters *(C. virginica)* were induced to spawn in filtered seawater at a temperature of 27-29 C. The parent stocks used for the 2 growth experiments were not the same, but were both conditioned in a flume with water at 20-22 C for 4-8 weeks and supplementally fed with the alga *Tetraselmis suecica*. Gametes were collected and fertilized. About 12.5×10^6 fertilized eggs were placed in a 250 l fiberglass larval tank and allowed to develop to straighthinge larvae. When the larvae reached the straighthinge stage (18 to 24 hrs after fertilization at 27-29 C), larval cultures were divided into 4 fiberglass larval tanks at a larval density of 5 to 6 larvae/ml. Two tanks of larvae were fed with an algal diet (a combination of *Pavlova (Monochrysis) lutheri* and *Isochrysis galbana)* until the larvae reached the "eyed" stage. The algae, *P. lutheri* and

I. galbana, were cultured as described previously (9). Algal batch cultures in log growth phase (4-15 days old) of *P. lutheri* and *I. galbana* were used as larvel food. Equal numbers of cells of each algal species were mixed and fed to the larvae. Algal cells for lipid analysis were harvested during log growth phase and lyophylized prior to total lipid extraction. The other 2 tanks of larvae were starved for the same period and served as control. Seawater at 27-29 C was filtered through 10 and 1 μm Cuno filters and used throughout the experiment. No attempt was made to collect any residual bacteria or other particulate matter in the seawater for fatty acid analysis.

Larval samples were collected from both groups (algae fed and "starved") on every other day up to the "eyed" stage. Larval samples from either group were pooled into a 1 or 2 l beaker and the number of larvae determined. Algae-fed larvae were maintained in the beaker for 4 to 6 hrs to allow the larvae to clear their guts. Larvae from each group were divided into 2 fractions, one-half used for estimation of total neutral lipid and the other half for fatty acid composition determination. The larvae were washed with isotonic ammonium formate (0.9% NH_4COOH) to remove the sea water. Larvae were then placed in 13 x 100 mm glass tubes, freeze-dried, flushed with nitrogen and stored at -20 C. Total lipid extraction was performed on the dried larval samples between 2 weeks and 3 mos.

Larvae from growth Experiment I were used to determine the fatty acid composition of neutral and polar lipid fractions. Larval samples from Experiment II were used to determine the fatty acid composition of the total lipid and total neutral lipid of the larvae.

Lipid and Fatty Acids Analyses

Total lipids were extracted with chloroform, methanol and distilled water (2:2:1) according to the procedure of Bligh and Dyer (10). Lipid classes were separated on thin layer chromatography (TLC), pre-coated silica gel plated (Applied Science, U.S.A.), using a solvent mixture of hexane, ether and acetic acid (85:15:1, v/v). The TLC plates were developed twice. Neutral and polar lipid bands were scraped off the plate and recovered with chloroform and methanol (2:1, v/v). Total neutral lipid, in terms of triacylglycerol, was quantitated in triplicate samples by a colorimetric method (11).

For fatty acid analysis, the total, neutral and polar lipids recovered from TLC subsequently were transesterified with BF_3 in CH_3OH (14%, v/v) with additional CH_3OH and benzene (12). Impurities also were removed from the fatty acid methyl esters by TLC. Separation of fatty acid methyl esters (FAME) was carried out on a Varian Model 3700 gas chromatograph with a packed column (2.4 m x 4 mm id glass column, 3% EGSP-Z on 100/120 mesh Gas-Chrom Q). The column was temperature-programmed from 100-190 C at 8 C/min. The flow rates of nitrogen, compressed air and hydrogen were 40 ml/ min, 300 ml/min and 30 ml/min, respectively. Fatty acids were identified using commercially available standards (Applied Science, U.S.A.), a secondary reference standard (fatty acid methyl esters of cod liver oil [13]) and graphic techniques (14). The chromatograms were quantitated by triangulation techniques.

RESULTS AND DISCUSSION

P. lutheri and *I. galbana* are species used in traditional algal diets (15-19) for oyster larvae. Usually, larvae fed on these algae would develop to the "eyed" stage in 10 to 15 days, as they did in this study.

In growth Experiment I, the larvae reached the "eyed" stage in 10 days, whereas in growth Experiment II, it took 12 days. Changes take place in the appearance and structure of the larva as it grows. "Eyed" larvae (pediveligers) are free-swimming larvae at a stage of development ready to set (change from free-swimming to sedentary).

In terms of assimilation or metabolic interconversion, attention is focused on the concentrations of saturated, mono-unsaturated, polyunsaturated, ω3 and ω6 fatty acids. Weight percentage of saturated, monoethylenic, polyethylenic, ω3 and ω6 fatty acids in neutral, polar and total lipids of algal-fed and "starved" larvae are shown in Tables 1 and 4. Total saturated fatty acids are higher in the total lipid of "starved" larvae and in the neutral lipid of algal-fed larvae than in the polar lipid of algal-fed larvae. In contrast, the weight percentage of polyunsaturated fatty acids, especially in the older and eyed larvae, are consistently higher in the polar lipid than in the neutral and total lipid of the algal-fed larvae or in the total lipid of the "starved" larvae. Similarly, in algal-fed larvae, the polar lipid had higher levels of ω3 fatty acids than in the neutral lipid (Table 1 and 2, Fig. 1). Phospholipids are the structural components of the cell membranes. These results reinforce the importance of polyunsaturated fatty acids (PUFA), especially long chain PUFA, in the physiology of marine animals for processes such as membrane integrity and permeability. Conservation of PUFA in the phospholipid fraction of the ocean-strider, *Halobates fijiensis*, during starvation for 8 days has been reported (20). The higher weight per-

TABLE 1

Weight Percentage of Saturated, Monoethylenic, Polyethylenic, ω6 and ω3 Fatty Acids in the Total Lipid of the Algal Diet and Neutral Lipid of Oyster Larvae

	Algal food	Larvae					
Age (days)		1	3	6	8	10	10 (eyed)
Size (μm)		79	85	127	168	230	270
Fatty acids							
Saturated	49.0	59.0	41.9	34.3	50.0	62.3	44.0
Monoethylenic	24.5	21.2	18.5	30.4	18.8	11.7	20.7
Polyethylenic	18.0	17.6	6.91	31.2	24.7	19.2	28.0
20:5ω3	3.45	–	1.05	0.38	6.12	6.60	8.86
22:6ω3	3.24	–	–	0.34	7.85	3.33	6.94
Σω6	3.00	10.75	5.39	28.06	6.24	5.58	5.65
Σω3	15.0	6.41	1.52	2.92	18.6	13.6	22.4
ω6/ω3	0.20	1.67	3.54	9.61	0.34	0.41	0.25

"–" Means not detected (Growth Experiment I).

TABLE 2

Weight Percentage of Saturated, Monoethylenic, Polyethylenic, ω6 and ω3 Fatty Acids in the Total Lipid of the Algal Diet and Polar Lipid of Oyster Larvae

	Algal food	Larvae				
Age (days)		1	3	6	10	10 (eyed)
Size (μm)		79	85	127	230	270
Fatty acids						
Saturated	49.0	30.5	35.2	32.6	29.5	31.1
Monoethylenic	24.5	22.7	38.3	22.3	16.4	17.2
Polyethylenic	18.0	37.0	14.2	26.7	39.6	35.8
20:5ω3	3.45	7.27	0.85	3.81	7.46	4.56
22:6ω3	3.24	9.70	3.58	5.22	12.9	8.77
Σω6	3.00	4.16	4.79	9.30	7.15	9.24
Σω3	15.0	18.9	6.95	11.9	24.4	17.8
ω6/ω3	0.20	0.22	0.76	0.78	0.29	0.52

(Growth Experiment I).

centage of ω3 fatty acids in the phospholipid fractions than in the neutral lipids during larval development may be due to the selective insertion of ω3 series rather than the ω6 series in the phospholipid of the cell membrane. This biochemical phenomena has been suggested to be a characteristic mechanism of aquatic marine animals (21).

In the total lipid and the neutral lipid fractions the total 20:5ω3 and 22:6ω3, and the total ω3 fatty acids, increased with larval age (Tables 1 and 3, Fig. 1). Eicosapentaenoic acid (20:5ω3) and 22:6ω3 were not detected in either the total lipid and neutral lipid of young larvae (one-day and 3 day-olds) or in the total lipid of "starved" larvae (Tables 1, 3 and 4). These 2 fatty acid components, 20:5ω3 and 22:6ω3, have been reported to be essential for oyster spat *(Crasostrea gigas)* growth (22). The results of this study do indicate the accumulation of these 2 fatty acids in older larvae but not in younger and initial larvae (Tables 1 and 3). The accumulation of 20:5ω3, 22:6ω3 and total ω3 fatty acids (Fig. 1) in older larvae is consistent with 2 ideas: [1] the accumulation of these fatty acids is attributable to the food intake; since depot liquids (neutral lipids) are the primary energy reserve in the majority of marine animals (8,17,20,23,24) and the fatty acid composition of the neutral lipid is influenced by the diet, and [2] older larvae have some ability to biosynthesize long chain ω3 fatty acids de novo. DeMoreno et al. (25) reported that the yellow clam, *Mesodesma matroides*, is capable of elongating and desaturating linoleic and α-linolenic acids from ingested phytoplankton with the presence of dietary protein (casein hydrosylate). Similarly, Waldock and Holland (26) reported

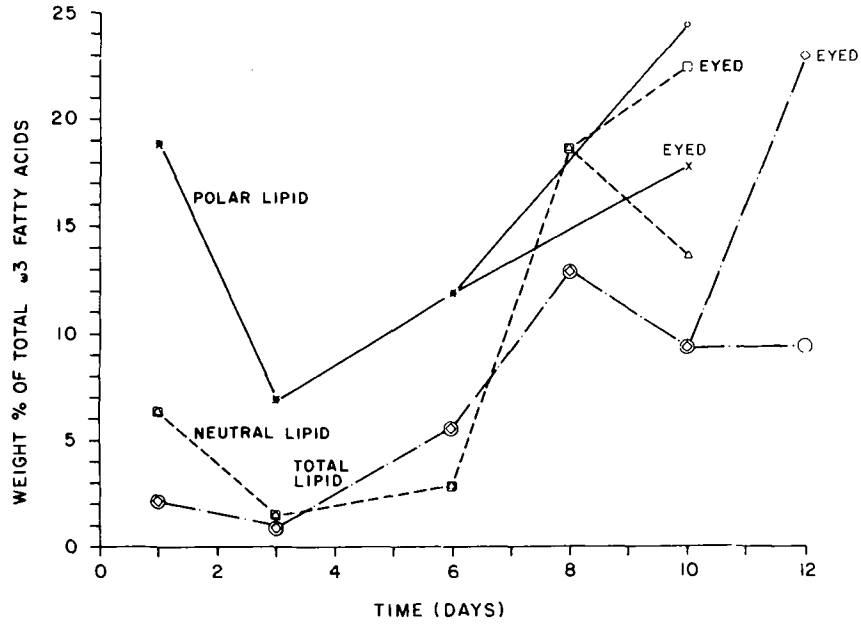

FIG. 1. Weight percentage of total ω3 family fatty acids in different lipid fractions of oyster larvae during development (from straight-hinge stage to the "eyed" stage).

TABLE 3

Weight Percentage of Saturated, Monoethylenic, Polyethylenic, ω6 and ω3 Fatty Acids in the Total Lipid of the Algal Diet and Total Lipid of Oyster Larvae

	Algal food	Larvae						
Age (days)		1	3	6	8	10	12	12 (eyed)
Size (μm)		73	87	138	182	238	270	327
Fatty acids								
Saturated	49.0	63.8	81.3	67.1	46.2	54.1	53.2	35.3
Monoethylenic	24.5	18.3	5.66	20.9	29.5	17.6	24.8	29.8
Polyethylenic	18.0	5.25	3.53	9.10	18.5	15.4	14.6	31.2
20:5ω3	3.45	–	–	1.96	4.43	2.86	2.83	6.96
22:6ω3	3.24	–	–	1.27	3.23	3.47	3.59	9.40
Σω6	3.00	2.93	2.51	1.97	3.45	4.54	3.78	5.95
Σω3	15.0	2.23	1.02	5.63	12.9	9.35	9.43	22.9
ω6/ω3	0.20	1.31	2.46	0.35	0.27	0.49	0.40	0.26

"–" Means not detected (Growth experiment II).

some incorporation of ^{14}C-labeled dietary fatty acid into C20 and C22 mono- and polyunsaturated fatty acids, including 20:5ω3 in juvenile oysters of *C. gigas*. In the neutral and total lipid fractions, higher weight percentage of PUFA and ω3 fatty acids were observed in eyed than pre-eyed prepediveliger larvae (Table 5). This suggests that in order to metamorphose, the larvae require a relatively higher level of PUFA and ω3 fatty acids reserved in the depot fats for energy provision and conservation of structural PUFA in phospholipids.

The "starved" larvae had a higher percentage of saturated fatty acids than the fed larvae, but lacked 20:5ω3 and 22:6ω3 fatty acids. Similar observations were reported by DeMoreno et al. (25) for clams in a season in which less food was available, and the food source was mainly detritus. Eicosenoic acid (20:1) and docosenoic (22:1) were detected in both "starved" and algal-fed larvae but not in the algal diet. Apparently, oyster larvae have the ability to elongate dietary 16:1 and 18:1 to 20:1 and 22:1. Alternatively, there may be a significant input of

TABLE 4

Weight Percentage of Saturated, Monoethylenic, Polyethylenic, ω6 and ω3 Fatty Acids in the Total Lipid of Starved Oyster Larvae

	Larvae					
Age (days)	1	3	6	8	10	12
Size (μm)	73.2	81.0	95.5	102.0	113.2	119.4
Fatty acids						
Saturated	72.1	63.3	80.9	78.8	79.4	64.5
Monoethylenic	19.4	11.7	9.29	7.22	7.38	7.47
Polyethylenic	5.96	17.7	5.93	12.5	9.76	13.3
20:5ω3	–	–	–	–	–	–
22:6ω3	–	–	–	–	–	–
Σω6	2.93	11.7	5.93	9.29	5.25	13.3
Σω3	2.23	5.97	–	1.48	3.36	–
ω6/ω3	1.31	1.95	–	6.25	1.56	–

"–" Means not detected or cannot be calculated (Growth experiment II).

TABLE 5

Total ω3, ω6 and Polyethylenic Fatty Acids in "Eyed" and Non-eyed Larvae of the Same Age

		Fatty acids					
Lipid fraction	Growth experiment	Polyethylenic Non-eyed	Eyed	ω6 Non-eyed	Eyed	ω3 Non-eyed	Eyed
Neutral	I	19.2	28.0	5.58	5.65	13.6	22.4
Polar	I	39.6	35.8	7.15	9.24	24.4	17.8
Total	II	14.6	31.2	3.78	5.95	9.43	22.9

monounsaturated fatty acids from dissolved organic material, detritus or bacteria. The considerably higher ration of ω6/ω3 fatty acids in oyster larvae compared to algal diets and the deposition of ω6 fatty acids in "starved" larvae also suggests that this fatty acid comes from either the dissolved or particulate phase of the seawater.

During larval development, there was a marked increase of neutral lipid (triacylglycerol) (from 3.6 to 11.1 × 10^{-3} μg larva^{-1}) in larvae up to the pre-eyed stage. In contrast, the neutral lipid content in the "starved" larvae remained relatively constant and low (3.6 to 1.89 × 10^{-3} μg larva^{-1}). The apparent accumulation of a reserve of neutral lipid (triacylglycerol) during larval development agrees with the previous studies by Holland and Spencer (2). They proposed that lipid, especially neutral lipid (triacylglycerol), is the main energy reserve during development through metamorphosis. This hypothesis may apply for larvae of many benthic marine invertebrates. Triacylglycerol reserves are accumulated during the larval stages and provide energy for metamorphosis into the adult stage (8). The amount of triacylglycerol in "eyed" larvae (6.9 × 10^{-3} μg larvae^{-1}) was much lower than in pre-eyed larvae (11.1 × 10^{-3} μg larvae^{-1}). The decline of neutral lipid (triacylglycerol) in "eyed" larvae is probably due to the consumption of neutral lipid for the development of new organs: eyes, foot and related structures. The presence of an "eye" in the free-swimming larvae indicates the approaching settlement and metamorphosis. The accumulation of a neutral lipid reserve for development may no longer be essential since after settlement the principal energy reserve is carbohydrate (glycogen).

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REFERENCES

1. Millar, R.H. and Scott, J.M. (1967) J. Mar. Biol. Assoc. U.K. 47, 475-484.
2. Holland, D.L., and Spencer, B.E. (1973) J. Mar. Biol. Assoc. U.K. 53, 287-298.
3. Creekman, L.L. (1977) M.A. Thesis, University of Virginia, Charlottesville, Virginia.

4. Waldock, M.J., and Nascimento, I.A. (1979) Mar. Biol. Lett. 1, 77-86.
5. Chu, F.L., and Dupuy, J.L. (1980) Lipids 15, 356-364.
6. Gallager, S.M., and Mann, R. (1983) Abstract. 75th Annual National Shellfisheries Association meeting, Hilton Head Island, South Carolina, June 6-9, 1983.
7. Helm, M.M., Holland, D.L., and Stephenson, R.R. (1973) J. Mar. Biol. Assoc. U.K. 53, 673-684.
8. Holland, D.L. (1978) in Biochemical and Biophysical Perspectives in Marine Biology, (Malins, D.C., and Sargent, J.R., eds) Vol. 4, pp. 85-123, Academic Press, London and New York.
9. Chu, F. -L. E., Dupuy, J.L., and Webb, K.L. (1982) Aquaculture 29, 241-252.
10. Bligh, E.G., and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911-917.
11. Heath, J.R., and Barnes, H. (1970) J. Exp. Mar. Biol. Ecol. 5, 199-233.
12. Morrison, W.R., and Smith, L.M. (1974) J. Lipid Res. 5, 600-608.
13. Ackman, R.G. (1965) J. Am. Oil Chem. Soc. 42, 38-42.
14. Ackman, R.G. (1963) J. Am. Oil Chem. Soc. 40, 558-564.
15. Ukeless, R. (1969) in Proceedings of the Conference on Artificial Propogation of Commercially Valuable Shellfish (Price, K.S., and Maurer, D.L., eds.) pp. 43-64, College of Marine Studies, University of Delaware, Newark, Delaware.
16. Walne, P.R. (1963) J. Mar. Biol. Assoc. U.K. 43, 767-784.
17. Walne, P.R. (1970) Fish. Invest. Ser. II, Mar. Fish. G.B. Minist. Agric. Fish. Food 26, 1-62.
18. Davis, H.C. (1953) Biol. Bull. 104, 334-350.
19. Davis, H.C., and Guillard, R.R. (1958) Fish Bull. 58, 293-304.
20. Holland, D.L., Crisp, D.J., and East, J. (1983) Mar. Biol. Letters 4, 259-265.
21. Cowey, C.B., and Sargent, J.R. (1979) in Fish Physiology (Hoar, W.S., and Randall, D.J., eds.) pp. 38-48, Academic Press, New York.
22. Langdon, C.J., and Waldock, M.J. (1981) J. Mar. Biol. Assoc. U.K. 61, 431-448.
23. DeMoreno, J.E.A., Moreno, V.J., and Brenner, R.R. (1976) Lipids 11, 334-340.
24. Sargent, J.R. (1976) in Biochemical and Biophysical Perspectives in Marine Biology (Malins, D.C., and Sargent, J.R., eds.), Vol. 3, pp. 149-212, Academic Press, New York.
25. DeMoreno, J.E.A., Moreno, V.J., and Brenner, R.R. (1976) Lipids 11, 561-566.
26. Waldock, M.J., and Holland, D.L. (1984) Lipids 19, 332-336.

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Lipid, Sterol and Fatty Acid Composition of Antarctic Krill (*Euphausia superba* Dana)

H. FRICKE,[a] G. GERCKEN,[a] W. SCHREIBER[b] and J. OEHLENSCHLÄGER,[b,*]
[a]*Department of Biochemistry, University of Hamburg, Hamburg, and* [b]*Institute for Biochemistry and Technology in the Federal Research Centre of Fisheries, Palmaille 9, D-2000 Hamburg 50, Germany*

ABSTRACT

The lipid classes, fatty acids of total and individual lipids and sterols of Antarctic krill (*Euphausia superba* Dana) from two areas of the Antarctic Ocean were analyzed by thin layer chromatography (TLC), gas liquid chromatography (GLC) and gas liquid chromatography/mass spectrometry (GLC/MS). Basic differences in the lipid composition of krill from the Scotia Sea (caught in Dec. 1977) and krill from the Gerlache Strait (caught in Mar. 1981) were not observed. The main lipid classes found were: phosphatidylcholine (PC) (33-36%), phosphatidylethanolamine (PE) (5-6%), triacylglycerol (TG) (33-40%), free fatty acids (FFA) (8-16%) and sterols (1.4-1.7%). Wax esters and sterol esters were present only in traces. More than 50 fatty acids could be identified using GLC/MS, the major ones being 14:0, 16:0, 16:1(n-7), 18:1(n-9), 18:1(n-7), 20:5(n-3) and 22:6(n-3). Phytanic acid was found in a concentration of 3% of total fatty acids. Short, medium-chain and hydroxy fatty acids ($C \leq 10$) were not detectable. The sterol fraction consisted of cholesterol, desmosterol and 22-dehydrocholesterol.
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INTRODUCTION

Krill (*Euphausia superba* Dana) lives exclusively in cold Antarctic waters and is the central link in the Antarctic food web. Its general chemical and biochemical composition has been the subject of several investigations (1). A number of contributions also have dealt with the lipid content and lipid composition of this pelagic euphausiid. Lipid contents between 1% and 6% have been published (2), and remarkably differing data have been reported for lipid composition (3-12). The main lipid classes found by almost all investigators were phosphoglycerolipids, triacylglycerols (TG), free fatty acids (FFA) and free sterols. The dominating fatty acids reported were 16:0 among saturated fatty acids and 18:1, 20:5 and 22:6 among unsaturated and polyunsaturated fatty acids. This investigation has been carried out to give thorough and complete analyses of lipid classes, fatty acids and sterols, supported by mass spectrometry (MS).

MATERIALS AND METHODS

Sample Collection and Preparation

Antarctic krill were collected from the Scotia Sea on December 16, 1977 at 57° 47′ S; 42° 43′ W (13) and from the Gerlache Strait on March 12, 1981 at 64° 33.7′ S; 62° 32′ W (14) during the second (1977/78) and third (1980/81) Antarctic expeditions of the Federal Republic of Germany with FMS "Julius Fock" and FRV "Walther Herwig," respectively, using a 1219 mesh pelagic Krill net.

Krill samples of 5 kg were quick-frozen and stored at −35 C until analyzed. Subsamples prepared from the core of the 5 kg samples were homogenized in a mortar under liquid nitrogen, and lipid extraction was performed according to Folch et al. (15). Lipids were dissolved in dichloromethane: methanol 1:1 (v/v) and stored under a nitrogen atmosphere at −23 C.

Thin Layer Chromatography and Gas Liquid Chromatography

Crude lipids were separated into classes by TLC on HPTLC-plates (E. Merck, Darmstadt) developed with n-hexane:diethylether:glacial acetic acid 60:40:1 (v/v) for neutral lipids, and with dichloromethane:methanol:glacial acetic acid 60:30:10 (v/v) or dichloromethane:methanol:aqueous ammonia 60:20:5 (v/v) for polar lipids. Lipid classes were visualized by exposure to iodine vapor or by charring with 50% sulphuric acid. After 2 dimensional TLC using the above mentioned solvents, identification was achieved by comparison with standard mixtures and lipid class specific stainings (16). After the silica gel was scraped off, the eluted acylglycerols were quantified by an enzymatic test for esterified glycerol (E. Merck, Darmstadt), and phosphoglycerides by phosphorus determination (17). FFA and sterols were determined by GLC using heptadecanoic acid and stigmasterol, respectively, as internal standards.

*To whom correspondence should be addressed.

Fatty acid methyl ester (FAME) of total lipids and individual lipid classes were prepared with 14% boron trifluoride in methanol (18), and fatty acid benzyl esters (FABE) according to Klemm et al. (19). Trimethylsilylation of sterols was carried out as described by Ballantine et al. (20). FAME and FABE were purified by TLC prior to GLC analysis. Separations and identifications were carried out on a polar wall coated (WCOT) open-tubular glass column (25 m) coated with SILAR 10 C (Packard instruments), temperature programmed from 110 C to 210 C (3 C/min) and on a 50 m fused silica column (WCOT) coated with CP SIL 5, temperature programmed from 100 C to 320 C (3 C/min) using a Packard 428 gas chromatograph equipped with a FID and a HP 3371 integrator. Helium was used as carrier gas at a flow of 1 ml/min with a split ratio of 100:1. The presence of plasmalogens and alkylglycerols was tested subsequent to hydrolysis using the procedure of Pugh et al. (21).

GLC/MS analysis of FAME and trimethylsilyl (TMS) sterols was performed on a HP 5985A quadrupole mass spectrometer, ionization energy 70 eV, ion source temperature 200 C, column: 25 m WCOT coated with CP SIL 5 (Chrompak), temperature programmed from 140 C to 280 C (4 C/min).

Individual FAME, FABE and TMS sterol peaks were identified by co-chromatography with standards, by comparison with calculated equivalent chain length (ECL) values (22) and by mass spectra. To ensure identification of unusual fatty acids, samples were hydrogenated and rechromatographed. For positional analysis, cleavage of PC and PE was performed with phospholipase A_2 from *Crotalus durissus terrificus* (Boehringer, Mannheim). After 24 hr incubation in diethylether and 0.1 M tris-buffer, the reaction mixture was separated by TLC into lysophospholipids and FFA.

RESULTS AND DISCUSSION

Lipid Content and Lipid Composition

The total lipid content and the lipid composition data of the 2 krill samples are given in Table 1. Although different lipid compositions have been published, there is general agreement as to the main lipid classes present in *Euphausia superba* (3-12). The krill caught in December 1977 has a lower fat content than the krill caught in March 1981. This increase in fat content during the catching season, which coincides with the sexual maturity (2) of krill, has been shown previously (14). Beginning with a low fat content of approx. 1% on a wet weight basis in November/December, the fat content increases to approx. 6% in March/April.

TABLE 1

Lipid Composition of Antarctic Krill (*Euphausia superba* Dana)

Sample	12/1977	3/1981
Total lipid content (% wet weight)	2.7 ± 0.2	6.2 ± 0.3
Phospholipids		
Phosphatidylcholine	35.6 ± 0.1	33.3 ± 0.5
Phosphatidylethanolamine	6.1 ± 0.4	5.2 ± 0.5
Lysophosphatidylcholine	1.5 ± 0.2	2.8 ± 0.4
Phosphatidylinositol	0.9 ± 0.1	1.1 ± 0.4
Cardiolipin	1.0 ± 0.4	1.6 ± 0.2
Phosphatidic acid	0.6 ± 0.4	
Neutral lipids		
Triacylglycerols	33.3 ± 0.5	40.4 ± 0.1
Free fatty acids[a]	16.1 ± 1.3	8.5 ± 1.0
Diacylglycerols	1.3 ± 0.1	3.6 ± 0.1
Sterols	1.7 ± 0.1	1.4 ± 0.1
Monoacylglycerols	0.4 ± 0.2	0.9 ± 0.1
Others[b]	0.9 ± 0.1	0.5 ± 0.1
Total	98.9	99.3

Data are expressed as wt % of total lipids and represent means ± standard deviation of 3 separate experiments.

[a]Probably mostly artifacts.

[b]Traces of lysophosphatidylethanolamine, phosphatidylserine, sphingomyelin, glycolipids, sterol esters, waxes and carotenoids were detected.

Euphausia superba is extremely rich in phospholipids (≥40% of total lipids) and TG (33 and 40% respectively of total lipids). While the relative content of phospholipids is similar in the 1977 and 1981 samples, the percentages of TG differ somewhat. This is in accordance with the previous results of our laboratories (23), which show that the relative phospholipid concentration will not change with varying total lipid contents. In other marine organisms an increase of total lipid content usually is caused by an increase of TG (24).

The sterol contents of 1.4% and 1.7% respectively of total lipids are in the range which has been reported (2,25) for Krill. These are very low values compared with those of Clarke (3), who found up to 16.9% sterols of total lipids in krill from South Georgia. This difference may be due to the methods. Clarke used densitometry (3) and our laboratory GLC.

In the 1977 sample the FFA content is about twice that of the 1981 sample. The high value could be caused by the longer storage time of the 1977 sample. A residual lipolytic activity against phospholipids exists even at temperatures of −30 C and below. Samples of

the same haul which were cooked on board immediately after hauling and stored under the same conditions showed a FFA content which was much lower, ranging from 1% to 3% of total lipids. This low FFA content of freshly caught krill also was confirmed by Ellingsen (11).

In addition, lysophosphatidylcholine, lysophosphatidylethanolamine, phosphatidylinositol phosphatidic acid, cardiolipin and mono- and diacylglycerols were detected, whereas phosphatidylserine, sphingomyelin, glycolipids, wax esters and sterol esters were present only in trace amounts. Wax esters were found by Bottino (8) in the euphausiid *Euphausia crystallorophias* but not in *Euphausia superba*. The composition of carotenoids was not investigated but had been analyzed by others (26-28).

Fatty Acid Composition of Total Lipids

The composition of the fatty acids of total lipids of *Euphausia superba* is similar to that of other marine crustaceans and some marine fishes (29) (Tables 2 and 3). The main fatty

TABLE 3

Branched Chain Fatty Acid Composition of Total Lipids of *Euphausia superba* Dana

Sample	M+	ECL	12/1977	3/1981
13:0 i	228	12.6	tr.	n.d.
14:0 i	242	13.6	0.05 ± 0.01	n.d.
15:0 i	256	14.6	0.19 ± 0.00	0.31 ± 0.15
15:0 ai	256	14.7	0.21 ± 0.01	0.24 ± 0.07
16:0 i	270	15.6	0.09 ± 0.03	0.10 ± 0.06
17:0 i	284	16.6	0.54 ± 0.05	0.20 ± 0.02
17:0 br[a]	284	16.4	tr.	0.09 ± 0.02
17:1 br	282	16.5	0.05 ± 0.03	0.11 ± 0.08
17:1 br	282	16.2	tr.	0.10 ± 0.05
18:0 i	298	17.6	tr.	0.10 ± 0.01
Phytanic[b] acid	326	17.7	2.82 ± 0.41	1.2 ± 0.43

Data are expressed as wt % of total fatty acids and represent means ± standard deviation of 3 separate experiments.

tr. = trace; n.d. = not detected; br. = branched; i = iso; ai = anteiso.

[a]Presumably 7-methylhexadecanoic acid.

[b]3,7,11,15-tetramethylhexadecanoic acid.

TABLE 2

Fatty Acid Composition of Total Lipids of *Euphausia superba* Dana

Sample	M+[a]	ECL[b]	12/1977	3/1981	Sample	M+[a]	ECL[b]	12/1977	3/1981
10:0	186	10.0	tr.	tr.	18:4(n-3)	290	17.4	0.67 ± 0.07	0.62 ± 0.49
11:0	200	11.0	tr.	tr.	19:0	312	19.0	tr.	0.11 ± 0.16
12:0	214	12.0	0.23 ± 0.06	0.22 ± 0.06	19:1	310	18.8	0.12 ± 0.04	0.20 ± 0.09
13:0	228	13.0	0.04 ± 0.01	0.07 ± 0.04	19:2	308	18.7	tr.	0.07 ± 0.05
14:0	242	14.0	11.33 ± 1.48	15.23 ± 2.31	20:0	326	20.0	0.04 ± 0.00	0.19 ± 0.14
14:1	240	13.8	tr.	0.19 ± 0.01	20:1(n-7)	324	19.8	0.40 ± 0.01	0.50 ± 0.09
15:0	256	15.0	0.34 ± 0.01	0.27 ± 0.05	20:1(n-9)	324	19.7	0.77 ± 0.04	1.35 ± 0.23
15:1	254	14.8	tr.	0.04 ± 0.03	20:2	322	19.6	tr.	0.08 ± 0.06
16:0	270	16.0	25.91 ± 2.33	31.79 ± 1.73	20:4(n-3)	318	19.5	0.46 ± 0.10	0.22 ± 0.06
16:1(n-7)	268	15.7	7.26 ± 0.35	7.37 ± 0.34	20:5(n-3)	316	19.3	12.71 ± 1.57	7.83 ± 1.27
16:1(n-?)	268	15.8	0.09 ± 0.13	0.30 ± 0.01	21:0	340	21.0	tr.	tr.
16:2(n-6)	266	15.6	0.82 ± 0.01	0.12 ± 0.06	21:5(n-3)	330	20.2	0.42 ± 0.03	0.30 ± 0.18
16:3	264	15.5	tr.	0.29 ± 0.01	22:0	354	22.0	0.14 ± 0.03	tr.
16:4(n-3)	262	15.4	0.74 ± 0.06	0.48 ± 0.14	22:1(n-7)	352	21.6	0.29 ± 0.17	0.41 ± 0.16
17:0	284	17.0	0.06 ± 0.02	0.17 ± 0.15	22:1(n-9)	352	21.5	0.51 ± 0.06	1.22 ± 0.33
17:1	282	16.7	tr.	0.41 ± 0.05	22:5(n-3)	344	21.2	0.54 ± 0.09	0.24 ± 0.11
17:1	282	16.8	tr.	0.12 ± 0.06	22:5	344	21.4	tr.	0.04 ± 0.03
18:0	298	18.0	1.21 ± 0.18	2.14 ± 0.23	22:6(n-3)	342	21.1	5.41 ± 0.51	2.60 ± 0.79
18:1(n-7)	296	17.8	8.32 ± 0.54	7.49 ± 0.79	23:1	366	22.5	tr.	0.11 ± 0.07
18:1(n-9)	296	17.7	10.13 ± 2.20	10.52 ± 0.90	24:0	382	24.0	tr.	tr.
18:1(n-?)	296	17.9	tr.	0.09 ± 0.05	24:1	380	23.6	tr.	0.15 ± 0.11
18:2(n-6)	294	17.6	1.58 ± 0.09	0.74 ± 0.38	25:0	396	25.0	tr.	tr.
18:3(n-3)	292	17.6	0.47 ± 0.02	0.33 ± 0.07					
18:3(n-6)	292	17.3	0.21 ± 0.06	0.57 ± 0.35	Others[c]	–	–	3.95	2.45

Data are expressed as wt % of total fatty acids and represent means ± standard deviation of 3 separate experiments.

tr. = trace.

[a]M+: molecular weight of fatty acid methyl ester as determined by GLC/MS.

[b]ECL: equivalent chain length, calculated by plotting chain length (as carbon number) versus retention time on CP SIL 5.

[c]Predominantly branched chain fatty acids as given in Table 3 in detail.

TABLE 4

Fatty Acid Analysis of Polar Lipid Classes of *Euphausia superba* Dana

Polar lipid	PC		PE		LPC		PI		PA + Cl	
Sample	12/1977	3/1981	12/1977	3/1981*	12/1977	3/1981*	12/1977	3/1981*	12/1977	3/1981*
14:0	4.5 ± 1.1	2.8 ± 1.1	2.9 ± 1.1	–	9.1 ± 5.4	4.2	3.5 ± 0.3	3.2	6.0 ± 1.4	–
15:0	–	–	–	–	–	–	–	1.6	–	–
16:0	43.7 ± 7.2	25.7 ± 1.4	42.7 ± 9.3	24.2	40.5 ± 8.9	18.7	33.9 ± 5.9	24.9	39.3 ± 6.3	23.7
16:1(n-7)	3.7 ± 0.4	2.2 ± 0.3	2.0 ± 1.0	1.9	4.4 ± 2.3	2.8	2.2 ± 0.9	1.2	3.6 ± 0.8	4.3
18:0	1.8 ± 0.5	1.5 ± 0.2	3.2 ± 1.0	2.9	2.1 ± 0.3	1.5	6.1 ± 1.0	7.3	2.5 ± 0.1	2.6
18:1(n-7)	7.7 ± 0.8	6.1 ± 0.8	15.0 ± 3.9	16.3	9.7 ± 3.7	4.0	11.6 ± 3.3	10.9	12.3 ± 0.6	14.7
18:1(n-9)	9.2 ± 1.7	5.4 ± 1.1	5.4 ± 2.1	6.8	10.3 ± 3.3	7.3	6.5 ± 0.4	7.9	4.9 ± 1.5	8.7
18:2(n-6)	1.6 ± 0.1	1.1 ± 0.1	1.0 ± 0.6	1.0	1.1 ± 0.8	1.6	1.7 ± 0.7	1.7	1.4 ± 0.1	1.5
18:3(n-3)	–	0.8 ± 0.2	–	–	–	1.1	–	0.6	–	1.6
18:3(n-6)	–	2.7 ± 0.4	–	0.7	–	3.8	–	–	–	1.7
18:4(n-3)	–	–	–	–	–	–	–	–	–	0.8
20:1(n-7)	–	–	–	–	–	–	–	–	–	–
20:1(n-9)	0.6 ± 0.1	0.9 ± 1.1	–	0.8	–	0.8	–	1.1	–	1.1
20:5(n-3)	10.7 ± 0.6	29.9 ± 2.2	10.5 ± 4.9	21.1	2.6 ± 0.1	31.2	8.1 ± 0.1	20.1	1.9 ± 1.0	19.7
21:5(n-3)	1.0 ± 0.7	1.1 ± 0.0	–	0.7	–	1.6	–	1.9	–	0.8
22:1(n-7)	–	0.9 ± 0.1	–	–	–	1.0	–	–	–	–
22:1(n-9)	–	1.7 ± 0.2	–	–	–	1.5	–	1.4	–	–
22:5(n-3)	0.9 ± 0.6	0.6 ± 0.2	–	0.9	–	1.1	–	1.8	–	–
22:6(n-3)	6.2 ± 0.6	11.5 ± 1.0	7.6 ± 2.3	19.2	1.2 ± 0.2	12.2	1.8 ± 0.7	10.1	1.1 ± 0.3	15.5
Phytanic acid	0.7 0.6	–	–	–	–	–	–	–	–	–

Data are expressed as wt % of total fatty acids in one lipid class and represent means ± standard deviation of 3 separate experiments.

– = concentration of less than 0.5%, * = only one experiment performed.

PC = Phosphatidylcholine; PE = Phosphatidylethanolamine; LPC = Lysophosphatidylcholine; PI = Phosphatidylinositol; PA + Cl = Phosphatidic acid and Cardiolipin.

TABLE 5

Fatty Acid Analysis of Neutral Lipid Classes of *Euphausia superba* Dana

Neutral lipid	TAG		FFA		DG		MG		WE + SE	
Sample	12/1977	3/1981	12/1977	3/1981	12/1977*	3/1981*	12/1977*	3/1981*	12/1977*	3/1981*
12:0	0.5 ± 0.1	–	–	0.8 ± 0.2	–	–	–	–	3.7	–
14:0	23.3 ± 0.2	21.8 ± 2.0	7.9 ± 1.0	5.1 ± 0.7	4.5	6.1	2.1	3.8	14.8	8.8
15:0	0.5 ± 0.1	–	–	–	–	0.5	–	1.2	–	–
16:0	29.9 ± 1.6	21.8 ± 1.8	32.5 ± 1.1	12.1 ± 2.2	19.4	16.9	9.6	10.3	25.1	37.8
16:1(n-7)	8.9 ± 1.9	13.1 ± 0.3	4.8 ± 1.0	4.9 ± 0.5	5.6	7.1	2.0	6.6	10.8	8.8
18:0	1.5 ± 0.2	1.8 ± 0.3	1.5 ± 0.2	0.7 ± 0.1	2.1	2.0	–	2.1	2.2	2.6
18:1(n-7)	5.9 ± 1.1	6.6 ± 3.1	12.9 ± 2.7	8.5 ± 2.2	14.7	7.5	73.7	10.9	15.8	17.5
18:1(n-9)	11.9 ± 3.6	12.1 ± 2.5	7.1 ± 0.6	4.7 ± 1.3	6.5	10.4	2.3	14.5	14.3	11.9
18:2(n-6)	0.7 ± 0.5	1.0 ± 0.2	1.5 ± 0.3	0.9 ± 0.7	3.1	1.3	0.8	1.3	1.9	–
18:3(n-3)	–	–	–	0.7 ± 0.2	–	0.8	–	–	–	–
18:3(n-6)	–	4.1 ± 1.0	–	1.5 ± 0.7	0.7	3.0	–	1.8	–	–
18:4(n-3)	–	–	0.6 ± 0.2	3.5 ± 1.2	–	–	–	–	–	–
20:1(n-7)	0.5 ± 0.1	–	–	–	0.5	–	–	–	–	–
20:1(n-9)	0.8 ± 0.2	1.3 ± 0.0	0.5 ± 0.1	1.0 ± 0.3	0.8	0.8	–	0.6	–	–
20:5(n-3)	1.0 ± 0.1	3.3 ± 0.5	11.8 ± 2.2	30.0 ± 2.1	15.8	28.8	2.9	26.8	5.1	11.9
21:5(n-3)	–	–	0.5 ± 0.4	0.9 ± 0.2	–	0.7	–	1.4	–	–
22:1(n-7)	–	–	0.8 ± 0.3	–	2.0	–	–	–	–	–
22:1(n-9)	–	0.5 ± 0.2		0.9 ± 0.4	1.5	1.3	–	0.8	–	–
22:5(n-3)	–	–	0.5 ± 0.3	0.5 ± 0.1	2.5	–	–	1.0	–	–
22:6(n-3)	–	0.7 ± 0.2	6.3 ± 2.4	12.1 ± 1.5	7.0	8.2	1.7	12.8	–	–
Phytanic acid	5.6 ± 0.8	4.1 ± 0.6	1.5 ± 0.6	1.3 ± 0.7	1.5	1.6	1.4	1.3	0.8	0.7

Data are expressed as wt % of total fatty acids in one lipid class and represent means ± standard deviation of 3 separate experiments.

– = concentrations of less than 0.5%; * = only one experiment performed.

TAG = Triacylglycerols; FFA = Free Fatty Acids; DG = Diacylglycerols; MG = Monoacylglycerols; WE + SE = Wax Esters and Sterol Esters.

acids are 14:0 (11-15%), 16:0 (26-32%), 16:1(n-7) (7%), 18:1(n-9) (10%), 18:1(n-7) (8%), 20:5(n-3) (8-13%) and 22:6(n-3) (3-5%). Odd-numbered fatty acids with chain lengths ranging from C-11 to C-25 also were found in trace amounts and verified by GLC/MS. In the unsaturated fatty acids the species of the (n-3) series are dominant, while (n-6) fatty acids are found only to a limited extent. This also has been reported for marine shrimps and fish (29-31). Several branched-chain fatty acids ranging from C-13 to C-18 (straight-chain length) were found, most of them belonging to the iso- or anteiso series. Among the multi-branched-chain fatty acids phytanic acid (32-34), which was the main component, amounted up to 3% of total fatty acid content. The samples from the early season 1977 contain more unsaturated fatty acids, especially 20:5(n-3) and 22:6(n-3), and less saturated fatty acids such as 14:0 and 16:0 than the sample from March 1981. This difference in the fatty acid compositions seems to be a seasonal phenomenon which also was reported by Shibata (2).

In most of the investigations of krill lipids the fatty acids were determined only by their retention behavior (3,35). In this study it was possible to determine the mass, and hence the chain length and number of double bonds, for all fatty acids by the combination of GLC/MS. The number of 57 analyzed fatty acids exceeds that reported by Golovnya et al. (36), who used the same technique. According to their ECL values 20:1 and 22:1 belong to the (n-7) and (n-9) series and not to the (n-11) series (36). The data found suggest that a (n-7) monoene series is present carrying from 16:1(n-7) through 18:1(n-7) and 20:1(n-7) to 22:1(n-7) (37,38). Arachidonic acid which was found by Clarke (3), Golovnya (36) and Bottino (5) in krill, and by Bottino in a shrimp (39) as a minor component, was not found. Dembitskii (40) showed that marine crustacea contained high levels of lipids with alkenyl side chains. In the samples investigated neither free aldehydes nor dimethylacetals after derivatization could be detected. Short chain, medium chain and hydroxy fatty acids (≤C-12) were not detectable even after transesterification to the corresponding benzyl esters (19).

Fatty Acid Composition of Lipid Classes

The analysis of fatty acids of individual lipid classes indicates different fatty acid compositions for phospholipids (Table 4) and TG (Table 5). Fatty acids in TG are mostly saturated or monounsaturated with 14:0, 16:0, 16:1(n-7), 18:1(n-7) and 18:1(n-9) as dominating species. Polyunsaturated fatty acids were found only in small amounts.

In phospholipids phytanic acid was detected only in traces, but it represented 5.6% of TG fatty acids. The phospholipids and FFA have 16:0, 20:5(n-3) and 22:6(n-3) as principal fatty acids. In the individual lipid classes a difference can be seen between the December samples (1977) and the March samples (1981). The lipid classes of the December samples contain more saturated fatty acids and less unsaturated fatty acids than the March 1981 samples. The discrepancy in the seasonal changes of the fatty acid composition of total lipids as mentioned above and that of the individual lipid classes is caused by the different lipid class composition with varying relative amounts of TG.

The positional analysis of the fatty acids in the main phospholipids PC and PE (Table 6) shows that saturated fatty acids are commonly linked to the *sn*-1 position and that the *sn*-2 position is preferred by unsaturated fatty acids. In this respect krill has the same fatty acid distribution as other marine animals (41).

TABLE 6

Fatty Acid Positional Analysis in Phosphatidylcholine (PC) and Phosphatidylethanolamine (PE) of *Euphausia superba* Dana (1977 Sample)

Phospholipid	PC		PE	
sn-position	*sn*-1	*sn*-2	*sn*-1	*sn*-2
14:0	3.5	2.3	0.7	0.8
16:0	60.8	4.7	45.4	3.3
16:1(n-7)	1.5	5.4	0.7	0.5
18:0	1.9	0.8	5.6	1.5
18:1(n-7)	11.1	tr.	24.0	0.9
18:1(n-9)	3.5	22.0	4.8	2.9
18:2(n-6)	0.6	4.8	0.6	0.6
20:5(n-3)	5.6	27.7	5.7	31.3
22:6(n-3)	2.1	11.1	3.5	41.3
Others	9.4	21.2	9.0	16.9

Data are expressed as wt % of fatty acids in one position from one experiment.

TABLE 7

Composition of the Free Sterol Fraction in *Euphausia superba* Dana

Sample	12/1977	3/1981
Cholesterol[a]	70.0 ± 5.9	75.5 ± 3.7
Desmosterol[b]	18.2 ± 1.4	17.7 ± 1.1
22-Dehydrocholesterol[c]	11.5 ± 4.8	6.0 ± 3.5
Others	0.8 ± 0.5	1.0 ± 0.7
Total	100.5	100.2

[a]Cholesta-5-en-3β-ol

[b]Cholesta-5,24-dien-3β-ol

[c]22-*cis*/*trans*-cholesta-5,22-dien-3β-ol

Sterols

The krill samples contained 3 sterols as major components identified by GLC/MS and traces of other sterols and sterol esters with unknown structure. The proportions of cholesterol, desmosterol and 22-dehydrocholesterol are given in Table 7. Cholesterol, which cannot be synthesized de novo in marine crustaceans (42), is the main sterol. Desmosterol and 22-dehydrocholesterol levels are very high. These sterols are assumed to be intermediates in the conversion of dietary sterols to cholesterol (42, 43). A small amount of 22-dehydrocholesterol, but no desmosterol, also was detected in the Arctic euphausiid *Meganyctiphanes norvegica,* whereas the herbivorous copepod *Calanus finnmarchicus* contained 14.1% 22-dehydrocholesterol and 27.7% desmosterol besides cholesterol as main sterol (44).

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REFERENCES

1. Grantham, G.J. (1977) Southern Ocean Fisheries Survey Progr. GLO/SO/77/2, FAO, Rome.
2. Shibata, N. (1983) Bull. Jap. Soc. Sci. Fish. 49, 259-264.
3. Clarke, A. (1980) J. Exp. Mar. Biol. Ecol. 43, 221-236.
4. Rzavskaja, F.M., Sakaeva, E.A., and Dubrovskaja, T.A. (1979) Rybnoe chozjajstvo 1979(10), 53-54.
5. Bottino, N.R. (1973) Fed. Proc. Fed. Am. Soc. Exper. Biol. 32, 562.
6. Bottino, N.R. (1974) Mar. Biol. 27, 197-204.
7. Bottino, N.R. (1975) Comp. Biochem. Physiol. 50 B, 479-484.
8. Pierce, R.W., van der Veen, J., and Olcott, H.S. (1969) J. Agr. Food Chem. 17, 367-369.
9. van der Veen, J., Medwadowski, B., and Olcott, H.S. (1971) Lipids 6, 481-485.
10. Nonaka, J., and Koizumi, C. (1964) Bull. Jap. Soc. Sci. Fish. 30, 630-634.
11. Ellingsen, T.E. (1982) Biokjemiske studier over antarktisk krill, Ph.D. Thesis, University of Trondheim, 239-316.
12. Mori, M., and Hikichi, S. (1976) The Report of the Central Res. Lab. of Nippon Suisan Co., Ltd. No. 11, 11-17.
13. Hempel, G., Sahrhage, D., Schreiber, W., and Steinberg, R. (1979) Arch. FischWiss. 30 (Beih. 1), 1-119.
14. Christians, O., Birnbaum, A., Leinemann, M., Manthey, M., and Oehlenschläger, J. (1982) Arch. FischWiss. 33 (Beih. 1), 143-170.
15. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497-509.
16. Kates, M. (1972) Techniques of Lipidology, North-Holland Publishing Company, Amsterdam.
17. Broekhuyse, R.M. (1968) Biochim. Biophys. Acta 152, 307-315.
18. Morrison, W.R., and Smith, L.M. (1964) J. Lipid Res. 5, 600-608.
19. Klemm, H.P., Hintze, U., and Gercken, G. (1973) J. Chromatogr. 75, 19-27.
20. Ballantine, J.A., Roberts, J.C., and Morris, R.J. (1980) J. Exp. Mar. Biol. Ecol. 47, 25-33.
21. Pugh, E.L., Kates, M., and Hanahan, D.J. (1977) J. Lipid Res. 18, 710-716.
22. Heckers, H., Dittmar, K., Melcher, F.W., and Kalinowski, H.D. (1977) J. Chromatogr. 135, 93-107.
23. Fricke, H., and Schreiber, W. (1983) Naturwiss. 70, 308-309.
24. Ackman, R.G. (1982) in Proceedings of the Second International Conference on Aquaculture Nutrition: Biochemical and Physiological Approaches to Shellfish Nutrition (Pruder, G.D., Langdon, C.J., and Conklin, D.E., ed.) Baton Rouge, Louisiana, pp. 358-376.
25. Kubota, K. (1980) J. Jap. Soc. Food Nutr. 33, 191-193.
26. Czeczuga, B., and Kłyszejko, B. (1978) Pol. Arch. Hydrobiol. 25, 657-662.
27. Czerpak, R., Jackowska, H., and Mical, A. (1980) Pol. Polar Res. 1, 139-145.
28. Yamaguchi, K., Miki, W., Toriu, N., Kondo, Y., Murakami, M., Konosu, S., Satake, M., and Fujita, T. (1983) Bull. Jap. Soc. Sci. Fish. 49, 1411-1415.
29. Ackman, R.G. (1980) in Advances in Fish Science and Technology (Connell, J.J., ed.) pp. 86-103, Fishing News Books Ltd., Farnham, Surrey, U.K.
30. Chanmugam, P., Donovan, J., Wheeler, D.J., and Hwang, D.H. (1983) J. Food Sci. 48, 1440-1441 and 1462.
31. Bell, M.V., Simpson, C.M.F., and Sargent, J.R. (1983) Lipids 18, 720-726.
32. Hansen, R.P. (1969) Aust. J. Sci. 32, 160-161.
33. Hansen, R.P., and Meiklen, S.M. (1970) J. Sci. Fd. Agric. 21, 203-206.
34. Ackman, R.G. (1968) Comp. Biochem. Physiol. 24, 549-565.
35. Ackman, R.G. (1970) J. Fish. Res. Bd. Canada 27, 513-533.
36. Golovnya, R.V., Kuzmenko, T.E., Samusenko, A.L., and Grigoreva, D.N. (1981) Appl. Biochem. Microbiol. 17, 47-53.
37. Ratnayake, W.N., and Ackman, R.G. (1979) Lipids 14, 795-803.
38. Ratnayake, W.N., and Ackman, R.G. (1979) Lipids 14, 804-810.
39. Lilly, M.L., and Bottino, N.R. (1981) Lipids 16, 871-875.
40. Dembitskii, V.M. (1979) Sov. J. Mar. Biol. 5(5), 86-91.
41. Brockerhoff, H., Yurkowski, M., Hoyle, R.J., and Ackman, R.G. (1964) J. Fish. Res. Bd. Canada 21, 1379-1384.
42. Morris, R.J., and Culkin, F. (1977) Oceanogr. Mar. Biol. Ann. Rev. 15, 73-102.
43. Gordon, T. (1982) J. Am. Oil Chem. Soc. 59, 536-545.
44. Sargent, J.R., and Falk-Petersen, S. (1981) Mar. Biol. 62, 131-137.

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Comparative Study of Lipogenic Enzymes in Several Vertebrates

NOBUKO IRITANI*, YUMIKO IKEDA, HITOMI FUKUDA and **AKIHIKO KATSURADA,**
Tezukayama Gakuin College, Sumiyoski-ku, Osaka 558, Japan

ABSTRACT

The liver lipogenic enzymes are compared among rats, chickens, frogs and fish. Although the apparent Km values of glucose-6-phosphate dehydrogenase for glucose-6-phosphate are not much different among all the species, those of malic enzyme for malate are much higher in chickens and fish than in rats and frogs. Glucose-6-phosphate dehydrogenase showed very high activities compared with malic enzyme in fish liver, and malic enzyme showed high activities in chicken liver. Although the apparent Km values of acetyl-CoA carboxylase and fatty acid synthetase for substrates are in the same range among all the animals, the activity of acetyl-CoA carboxylase seems to be extremely low in fish and frog livers, and that of fatty acid synthetase is low in frog livers only. In addition, the apparent Km values of α-glycerophosphate acyltransferase of fish liver are very high, and the enzyme activity appears to be extremely low compared to the others. Therefore, the enzymes at the first steps of both fatty acid and glycerolipid syntheses of poikilothermos animals appear to be very low. On the other hand, the Ouchterlony double-diffusion patterns showed that the lipogenic enzymes of chickens, frogs and fish are immunologically different from those of rats, with the exception of acetyl-CoA carboxylase in chickens. Therefore, it is suggested that the fatty acid and glycerolipid forming systems of poikilothermos animals are quite different from those of homoiothermos and the lipogenesis is very low in poikilothermos.
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INTRODUCTION

It has been reported that, while the hepatic lipogenic enzymes of fish respond to dietary manipulation similarly to those of rats, the length of time required to observe a change in enzyme activity was much longer than reported in rats (1,2). Fasting the fish for 6 days did not influence activities of lipogenic enzymes but the activities finally decreased after 23 days (2). As poikilothermos animals are capable of changing metabolic rates with temperature, the regulation of lipogenesis for storage of triglycerides may not be important for them. Therefore, poikilothermos animals may have different regulatory mechanisms from homoiothermos animals. In the present study, we have attempted kinetic and immunological analyses of lipogenic enzymes in poikilothermos animals such as fish and frogs, in comparison to homoiothermos animals such as rats and chickens.

MATERIALS AND METHODS

Male Wistar rats, 5 weeks old, were fed a stock diet (Oriental Shiryo No. MF, Japan) or a fat free diet (3) for 2 weeks before being killed. The stock diet contained 3.9% fat (53% linoleic acid, 23% oleic acid and 6.9% linolenic acid). Rats and frogs *(Bufo bufo japonica Schlegel)*, 3 mo old, were purchased from Awadzu Animals (Japan). Live fish (Sea bream, *Pagrus major Temminck et Schlegel)*, 2 yr old, were purchased from a fish market. Fresh livers of chicken *(Gallus gallus var domesticus Brisson)*, 2 mo old, fed on a stock diet (Oriental Shiryo N. PB, Japan) were obtained from a chicken slaughter house, immediately after decapitation. The stock diet for chicken contained 6% fat (39% oleic acid and 20% linoleic acid). Fish were naturally fed in the sea and frogs in a pond. All the animals were killed by decapitation between 8 a.m. and 10 p.m. The livers were removed quickly to measure lipid synthesis. Liver homogenate was prepared in 3 volumes of 0.25 M sucrose, using a Teflon glass homogenizer, and centrifuged at 10,000 × g for 10 min. The resultant supernatant was again centrifuged at 105,000 × g for 60 min (Spinco L-5 50, Beckman). The pellets (microsomes) and the supernatant were used for enzyme assays.

Fresh liver slices (0.3 g) were incubated for 120 min at 37 C with constant shaking in 2.5 ml of Krebs-Ringer phosphate buffer (pH 7.4), containing 1.6 mM sodium [1-^{14}C] acetate (0.1 μCi, purchased from The Radiochemical Centre, Amersham). Total lipids were extracted as described by Folch et al. (4) and separated by thin-layer chromatography (TLC) on silica gel H (Merck) impregnated with 10 mM Na_2CO_3. An aliquot of the lipid extract was developed with chloroform-methanol-acetic acid-acetone-water (200:40:40:80:20) for the separation of triglycerides, phospholipids and others. The lipid fractions were identified by comparison with authentic standards, which were visualized by exposure to iodine vapor. The silica gel zones corresponding to phospholipids and triglycerides were scraped and extracted with chloro-

*To whom correspondence should be addressed.

form:methanol (1:1). The radioactivity in the zones was measured in the scintillant solution. After saponification of an aliquot of the total lipids with 10% ethanolic KOH at 60 C for 1 hr, the aqueous phase was washed with petroleum ether and acidified. The fatty acids were extracted with petroleum ether. The radioactivities of the fatty acids in the petroleum ether were measured. Further, fresh liver slices (0.5 g) were incubated with tritiated water (50 μCi, purchased from New England) in 1 ml of the Krebs-Ringer phosphate buffer (pH 7.4). The total incorporation of the radioactivities into the total fatty acid fractions was measured according to the methods described above, without the separation of triglycerides and phospholipids.

Liver triglycerides and cholesterol were determined by the methods of Fletcher (5) and Zak et al. (6), respectively. The total phospholipid content was measured by phosphorus determination (7) in lipid fraction extracted from tissues according to Folch et al. (4).

Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) was assayed according to Glock and McLean (8) except the fish enzyme. The fish enzyme was assayed in 50 Mm Tris-HCl, pH 8, 0.1 mM glucose-6-phosphate, 40 mM $MgCl_2$ and 0.12 mM NADP. Malic enzyme (EC 1.1.1.40) was assayed according to Ochoa (9). Acetyl-CoA carboxylase (EC 6.4.1.2) activity was assayed at 37 C by the $H^{14}CO_3^-$-fixation method (10). α-Glycerophosphate acyltransferase (EC 2.3.1.15) were assayed according to the principle of Brandes et al. (11), using a 1 mM α-glycerophosphate and 1 mM palmityl-CoA. Diglyceride acyltransferase was assayed as described previously (12), essentially according to Weiss et al. (13). The optimum conditions of pH and additions in the assay mediums were examined for all the enzymes of the species of animals. Consequently, the enzymes were assayed by the methods described above, in linear ranges. Elongation and desaturation of fatty acids were determined by the methods of Podack et al. (14) and Mercuri et al. (15), respectively. All the enzyme assays were conducted at 37 C.

Acetyl-CoA carboxylase was purified from rat liver as described previously (16,17). Glucose-6-phosphate dehydrogenase and malic enzyme were purified from rat liver, essentially according to Matsuda and Yugari (18), and Rutter and Lardy (19), respectively. For further purification, each enzyme was chromatographed on an affinity column containing immobilized N^6-(6-aminohexyl)-adenosine-2′, 5′-bisphosphate and eluted specifically by NADP, essentially according to Yeng and Carrico (20). The antisera and the immunoglobulins of the enzymes of rat liver against rabbit were prepared as described previously (21). Ouchterlony double-diffusion analyses (22) performed with the use of antibodies against the enzymes gave single precipitation bands. The enzyme preparations of glucose-6-phosphate dehydrogenase, malic enzyme and acetyl-CoA carboxylase were the precipitate fractions of 105,100 x g liver supernatant resulting from 70%, 70% and 30% saturation with ammonium sulfate, respectively.

The partially purified enzymes were applied onto 4.8 ml of a 5-20% (w/v) linear sucrose gradient containing 50 mM Tris-HCl buffer (pH 8.0), 5 mM 2-mercaptoethanol and 1 mM EDTA. The tube was centrifuged in a Beckman SW-50.1 rotor at 25 C and 30,000 rpm for 16 hr. After centrifugation, fractions were collected and assayed for protein and enzyme activities. Details were similar to those described previously (23).

RESULTS AND DISCUSSION

Kinetic Study of Enzymes for Fatty Acid and Glycerolipid Syntheses

The results of the kinetic studies are summarized in Table 1. The Km values of glucose-6-phosphate dehydrogenase for glucose-6-phosphate in rat, chicken, frog and fish livers are in the same range. The Km values of malic enzyme for malate in fish and chicken livers are much higher than those in rats and frogs. The Km values of acetyl-CoA carboxylase for acetyl-CoA were similar among all the animals, although the Km values for citrate (activator of the enzyme) were lower in rats than in the others. The Km values of α-glycerophosphate acyltransferase for α-glycerophosphate and palmityl-CoA were similar among rat, chicken and frog livers. However, the Km values were very high in fish liver compared to the values of the others. The Km values of α-glycerophosphate acyltransferase for palmityl-CoA in fish liver was markedly higher than that in rat liver and the Km for α-glycerophosphate, also higher in fish liver. The Km values of the enzyme for other acyl-CoAs, such as linoleyl-CoA, oleyl-CoA and eicosapentaenoyl-CoA, were not much different from the value for palmityl-CoA (data not shown). The Km values of diglyceride acyltransferase for palmityl-CoA were similar among all the species.

Enzyme Activities for Fatty Acid and Glycerolipid Syntheses

Table 2 shows the enzyme activities for fatty acid and glycerolipid syntheses. Although the enzyme activities cannot be strictly compared

TABLE 1

Km Values of Hepatic Enzymes in Fatty Acid and Glycerolipid Syntheses for Substrates

Enzyme	Substrate or effector	Rat	Chicken	Frog	Fish
Glucose-6-phosphate dehydrogenase	(Glucose-6-phosphate)	83	83	77	77 μM
Malic enzyme	(L-Malate)	87	367	91	200
Fatty acid synthetase	(Malonyl-CoA)	50	51	15	50
Acetyl-CoA carboxylase	(Acetyl-CoA)	75	75	71	77
	(Citrate)	1.7	5.3	6.3	5.3 mM
α-Glycerophosphate acyltransferase	(α-Glycerophosphate)	200	200	227	667 μM
	(Palmityl-CoA)	20	20	15	500
Diglyceride acyltransferase	(Palmityl-CoA)	148	125	143	118

The enzymes were assayed as described in "Materials and Methods." The Km values of the enzymes for substrates or effector are shown.

The enzyme preparations of acetyl-CoA carboxylase and fatty acid synthetase were the precipitate fractions of 105,000 X g liver supernatant resulting 30% saturation with ammonium sulfate, and those of glucose-6-phosphate dehydrogenase and malic enzyme, the precipitate resulting 70% saturation.

Microsomal fractions of the liver homogenate were used for the assays of α-glycerophosphate acyltransferase and diglyceride acyltransferase.

The apparent Km values of the enzymes were obtained from Lineweaver-Burke plots.

among the animal species because of different nutritional conditions and metabolism, the relative activities of enzymes for fatty acid and glycerolipid syntheses can be compared in the same animals. It is well known that the activities of glucose-6-phosphate dehydrogenase, malic enzyme, fatty acid synthetase and acetyl-CoA carboxylase in the rat liver are decreased by fasting and markedly increased by a fat free diet. While the hepatic lipogenic enzymes of fish respond to dietary manipulation in a similar way, the length of time required to observe a change in enzyme activity was much longer than reported in rats (1,2). Fasting the fish for 6 days did not influence activities of lipogenic enzymes, but the activities decreased after 23 days (2). We also found that the enzyme activities for fatty acid and glycerolipid syntheses of fish and frog livers were not reduced by fasting for 7 days (data not shown). As the activities are not easily changed by dietary manipulation in frogs and fish, the hepatic enzyme activities for fatty acid and glycerolipid syntheses in commercially available frogs and fish are compared roughly to those in rats and chickens.

Although the activities of glucose-6-phosphate dehydrogenase and malic enzyme are comparable in rat liver, the malic enzyme activity is much higher than glucose-6-phosphate dehydrogenase activity in chicken liver. The malic enzyme activity in chicken is much higher than in the other species. Therefore, malic enzyme may be an important NADPH-donor in chicken liver, although the Km of malic enzyme for malate is very high. In fish, as glucose-6-phosphate dehydrogenase activity is very high, the enzyme may be an important NADPH-donor. It is notable that the activity of acetyl-CoA carboxylase is very low in poikilothermos animals such as frogs and fish. Further, the activity of fatty acid synthetase is also very low in frogs. In glycerolipid synthesis, α-glycerophosphate acyltransferase activity was extremely low in fish liver compared to the others, whereas diglyceride acyltransferase activities in all the species were at similar levels. In fish liver, as the Km values of α-glycerophosphate acyltransferase for α-glycerophosphate and acyl-CoA are very high (Table 1), glycerolipid synthesis does not seem to occur actively. Acetyl-CoA carboxylase and α-glycerophosphate acyltransferase react at the first steps of fatty acid and glycerolipid syntheses, respectively. These enzymes of poikilothermos animals seem to be different from those of homoiothermos at the first steps of the syntheses. Those are the limiting steps in many cases. As the activity of lipogenesis is very low in the poikilothermos animals, the regulation mechanisms for lipogenesis appear to be very poor. As poikilothermos animals are capable of changing metabolic rates with temperature, the regulation mechanism may not be important for them.

In Vitro Incorporation of [^{14}C] acetate or Tritiated Water into Lipids

The incorporation of [^{14}C] acetate into triglycerides and phospholipids by liver slices was in the same range among rats, chickens, frogs and fish, as shown in Table 3. Although the activities of acetyl-CoA carboxylase were very low in fish liver (Table 2), the acetate was incor-

TABLE 2

Enzyme Activities for Fatty Acid and Glycerolipid Syntheses in Liver

	Rat		Chicken	Frog	Fish
	Stock diet	Fat free diet	nmol/min/mg		
Glucose-6-phosphate dehydrogenase *1	25.8 ± 5.5	319 ± 22.1	6.15 ± 0.71	51.5 ± 1.50	121 ± 28.1
Malic enzyme *1	28.2 ± 1.58	189 ± 31.4	189 ± 18.3	25.0 ± 2.56	33.3 ± 1.28
Fatty acid synthetase *2	8.70 ± 0.30	22.3 ± 2.06	19.5 ± 3.33	2.01 ± 0.10	17.4 ± 2.41
Acetyl-CoA carboxylase *2	6.09 ± 0.54	22.9 ± 2.10	6.47 ± 0.17	0.08 ± 0.01	0.12 ± 0.03
α-Glycerophosphate acyltransferase *3	1.28 ± 0.13	1.56 ± 0.35	1.55 ± 0.36	1.68 ± 0.49	0.025 ± 0.008
Diglyceride acyltransferase *3	4.25 ± 0.58	3.22 ± 0.67	3.24 ± 0.69	5.23 ± 0.04	3.13 ± 1.58
Desaturase *3	0.60 ± 0.06				0.09 ± 0.02
Elongation *3	24.0 ± 3.21				8.57 ± 0.94

Mean ± SD (n=5-6). The animals used and the enzyme assays are explained in "Materials and Methods." The enzyme activities determined are discussed in "Results and Discussion."

Enzyme activities in the 105,000 × g supernatant fraction of liver homogenate, nmoles substrate utilized *1 or product formed *2 per min per mg protein at 37 C and in the microsomal fraction, nmoles product formed *3 per min per mg protein.

TABLE 3

Incorporation of [^{14}C]Acetate or Tritiated Water into Lipids by Liver Slices

	Rats[a]	Fish	Frog
		cpm X 10^{-3}/hr/g liver slices	
Incorporation of [^{14}C]acetate			
Triglycerides	2.98 ± 0.79	2.70 ± 0.81	2.51 ± 0.78
Phospholipids	3.80 ± 0.85	3.64 ± 0.91	4.17 ± 0.25
Fatty acids of total lipids	5.57 ± 0.96	5.33 ± 1.02	4.78 ± 0.83
Incorporation of tritiated water			
Fatty acids of total lipids	2.17 ± 0.28	2.39 ± 0.25	

Mean ± SD (n=3). The experiment was repeated six times for [^{14}C]acetate incorporation and twice for tritiated water, and one of the typical results for each incorporation is shown.

[a]Fed on a stock diet. Fresh liver slices were incubated at 37 C for 2 hr with constant shaking in Krebs-Ringer phosphate buffer, pH 7.4, containing either [^{14}C]acetate or tritiated water. Details are described in "Materials and Methods."

TABLE 4

Lipid Levels in Livers

	Rat		Chicken	Frog	Fish
	Stock diet	Fat-free diet	mg/g liver		
Triglycerides	20.5 ± 2.32	28.6 ± 3.59	101 ± 22.5	62.2 ± 10.6	255 ± 8.73
Phospholipids	15.4 ± 1.74	17.7 ± 1.73	15.0 ± 1.10	16.5 ± 1.84	14.2 ± 1.44
Cholesterol	3.04 ± 0.21	3.21 ± 0.24	4.14 ± 0.39	7.89 ± 1.38	4.27 ± 1.22

Mean ± SD (n=5-6). The animals used are explained in "Materials and Methods." The lipid level in livers of the animals not starved are shown.

porated into the fatty acids of triglycerides and phospholipids rather than into the glycerol. The comparable incorporations into fatty acids between rat and fish livers may be caused by the rapid decrease of [^{14}C]acetate level (for example, due to CO_2 formation) by rat liver slices during the incubation. However, the incorporations of tritiated water into total fatty acids of liver were also comparable between rat and fish. In any case, we found that the fatty acids are synthesized in fish liver.

On the other hand, Iritani et al. (24) previously reported that the rate of phospholipid synthesis in rat liver occurs at a roughly constant rate with dietary manipulation, whereas the rate of triglyceride synthesis varies greatly. Regardless of animal species (Table 3) and nutritional state, phospholipid synthesis appears to occur at a roughly constant rate.

Lipid Levels in Liver

As shown in Table 4, the contents of liver phospholipids in rats, chickens, frogs and fish were at a similar level. We previously reported that the phospholipid levels of rat liver did not vary with dietary manipulation (25). Therefore, regardless of animal species and nutritional states, phospholipid levels as well as synthesis rates (Table 3) appear to be roughly constant. The constant level of membrane phospholipids may be important for membrane function. The contents of liver cholesterol also were similar among rats fed a stock or a fat free diet, and chickens and fish. On the other hand, the triglyceride contents in liver change easily with nutritional and physiological states. For information, however, the triglyceride contents in the vertebrates also are shown in Table 4. The triglyceride contents of fish liver generally were very high. In any case, it is suggested that the major fatty acids of fish are not endogenous. Further evidence of the suggestion is that fish have a large amount of n-3 polyunsaturated fatty acids such as eicosapentaenoic acid and docosahexaenoic acid, which fish cannot synthesize (26-28).

Sucrose Density Gradient Centrifugation

The results of sucrose density gradient centrifugation of glucose-6-phosphate dehydrogenase showed that the sedimentations of the enzymes of chicken and frog are similar to

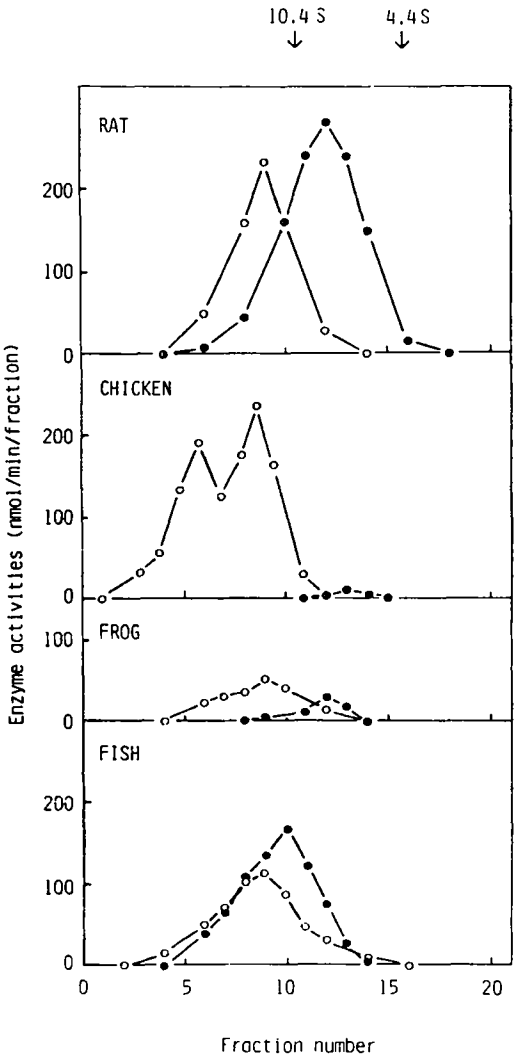

FIG. 1. Sucrose density gradient centrifugations of glucose-6-phosphate dehydrogenase and malic enzyme in rat, chicken, frog and fish livers. Sucrose density gradient centrifugation was carried out with a gradient from 5 to 20% (w/v) sucrose. The precipitate fractions of 105,000 × g liver supernatant from 70% saturation with ammonium sulfate were dialyzed and concentrated. Two-tenths ml of the enzyme solutions (containing 6-14 mg protein) was applied on the gradient (4.8 ml). The gradient tubes were centrifuged for 16 hr at 30,000 rpm and 4 C in a Beckman SW 50.1 rotor in a Spinco model L-5 ultracentrifuge. After centrifugation, 20 fractions were collected from each gradient. Fraction 0 is on the bottom of the tube, and the migration is from right to left. The enzyme activities of glucose-6-phosphate dehydrogenase (•) and malic enzyme (○) were measured as described in "Materials and Methods." Rabbit muscle pyruvate kinase [EC 2.7.1.40] ($S_{20,w}$ = 10.4 S) and horse hemoglobin ($S_{20,w}$ = 4.4 S) were used as external markers.

that of rat, whereas that of fish is larger (Fig. 1). The sedimentations of chicken, frog and fish malic enzyme coincided with that of rat, whereas chicken malic enzyme showed an extra peak of the sedimentation. On the other hand, Silpananta et al. (29) reported that malic enzyme of chicken liver showed a single symmetrical peak in the analytical ultracentrifuge, whereas the liver appeared to contain isozymes in polyacrylamide gel electrophoresis. It is known that a correlation between activity and sedimentation coefficient of mammalian acetyl-CoA carboxylase revealed some molecular properties of the active "large" form and the inactive "small" (30). As demonstrated previously (31), chicken acetyl-CoA carboxylase sedimented as a large form even in a medium without citrate (data not shown), whereas rat carboxylase sedimented as a small form in the absence of citrate and as a large form in its presence. Fish carboxylase sedimented as the large form in both the absence and the presence of citrate, similarly to chicken carboxylase (Fig. 2). However, the fish carboxylase was not activated with citrate and showed very little activity in both the absence and the presence of citrate.

Ouchterlony Double Diffusion Analysis

Ouchterlony double diffusion pattern showed that glucose-6-phosphate dehydrogenase, malic enzyme and acetyl-CoA carboxylase of chickens, frogs and fish are not immunologically homogenous to those of rats, except for chicken acetyl-CoA carboxylase, as shown in Fig. 3. It already has been reported that chicken carboxylase is homogenous to that of rats (32). The immunochemical analyses may reveal that glucose-6-phosphate dehydrogenase, malic enzyme and acetyl-CoA carboxylase of poikilothermos animals are quite different from those of rats, although the sedimentation behaviors in the sucrose density gradient centrifugation are similar.

In particular, the activities of acetyl-CoA carboxylase and α-glycerophosphate acyltransferase at the first steps of fatty acid and glycerolipid syntheses, respectively, were very low in poikilothermos animals. Furthermore, in fish liver, as the Km value of α-glycerophosphate acyltransferase for palmityl-CoA was extremely high, glycerolipid as well as fatty acid syntheses appear to be very low. However, when [1-^{14}C] acetate or tritiated water was incubated with fish liver slices, the incorporation of radioactivities into fatty acids of triglycerides and phospholipids was found. Although the major fatty acids of fish seem to be exogenous, a small amount of fatty acids may be synthesized in the liver. Lin et al. (1) reported that the major

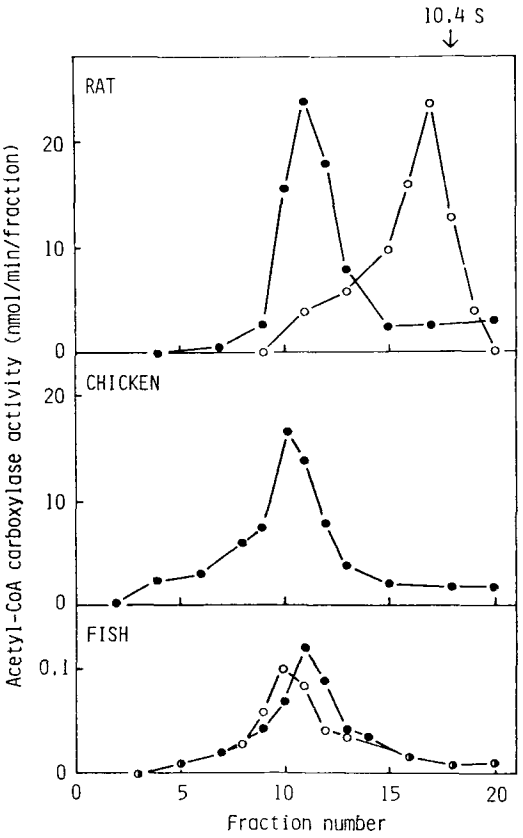

FIG. 2. Sucrose density gradient centrifugations of acetyl-CoA carboxylase in rat, chicken and fish livers. The precipitate fractions of 105,000 × g liver supernatant from 30% saturation with ammonium sulfate were dialyzed and concentrated. The enzyme solutions were incubated at 25 C for 10 min in a medium containing 50 mM Tris-HCl, pH 7.5, 5 mM 2-mercaptoethanol, 1 mM EDTA and 3 mg/ml bovine serum albumin, with (•) or without (○) 10 mM potassium citrate. Two-tenths ml of the incubated enzyme solutions (4.73-6.54 mg) was applied on 4.8 ml of sucrose gradient (5 to 20%, w/v) containing the same additions with the incubation medium. The gradient tubes were centrifuged for 80 min at 38,000 rpm and 25 C. Fraction 0 is on the bottom of the tube and the migration is from right to left.

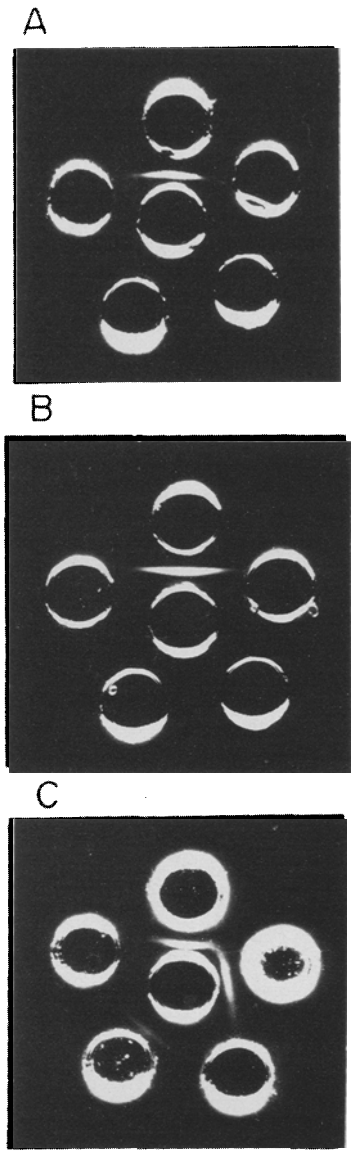

FIG. 3. Ouchterlony double-diffusion analyses of lipogenic enzymes. Agar gel (1%) contained 20 mM Tris-HCl buffer, pH 7.5 and 0.15 M NaCl. The enzyme preparations of glucose-6-phosphate dehydrogenase (A), malic enzyme (B) and acetyl-CoA carboxylase (C) were the precipitate fractions of 105,000 × g liver supernatant resulting from 70%, 70% and 30% saturation with ammonium sulfate, respectively. The center wells in A, B and C contained anti-γ-globulins of rat liver glucose-6-phosphate dehydrogenase, malic enzyme and acetyl-CoA carboxylase against rabbit (0.45, 0.22 and 0.36 mg, respectively). Top wells contained the corresponding enzyme preparations from rat liver (0.6-1.20 mg); upper right wells, from chicken (2.05-3.30 mg); lower right wells, from frog (3.11-4.40 mg); lower left wells, from chicken (2.05-3.30 mg); upper right wells, from fish (2.84-3.14 mg).

site of fatty acid synthesis in coho salmon appears to be the liver, and the fatty acid synthesis in adipose tissue is very low (similar to rats).

Kaul and Berdanier (33) found a circadian rhythm in glucose-6-phosphate dehydrogenase and, to a lesser degree, in malic enzyme. However, we observed no rhythm in glucose-6-phosphate dehydrogenase, malic enzyme and acetyl-CoA carboxylase (Fukuda, H., Katsurada, A., and Iritani, N., in preparation). As the enzyme

activities in a 24-hr rhythm were obtained from different animals, discrepancies in the results were found. As the half-lives of the enzymes of rat liver were mostly more than 2 days (34), our results appear to be possible. Also the half-life of malic enzyme of chicken liver was reported to be 55 hr (29). Therefore, the results shown in Table 2 do not seem to be influenced by circadian rhythm of food intake.

Glucose-6-phosphate dehydrogenase, malic enzyme and acetyl-CoA carboxylase of fish liver are immunologically different from the rat enzymes. Furthermore, the enzyme activities that represent the limiting steps for lipogenesis in rats are extremely low in fish. Consequently, it may be that the length of time required to observe changes in the enzyme activities is much longer in fish than in rats (1). As poikilothermos animals are capable of changing metabolic rates with temperature, the regulation of triglyceride synthesis for energy storage may not be important for them. Therefore, the regulatory mechanisms of lipogenesis appear to be very poor in poikilothermos animals. Although such a study suffers from a number of problems, information on comparisons of fatty acid and glycerolipid syntheses in several vertebrates is presented in this paper.

REFERENCES

1. Lin, H., Romsos, D.R., Tack, P.I., and Leveille, G.A. (1977) J. Nutr. 107, 1677-1682.
2. Lin, H., et al., (1977) J. Nutr. 107, 1477-1483.
3. Iritani, N., and Ikeda, Y. (1982) J. Nutr. 112, 2235-2239
4. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497-509.
5. Fletcher, M.J. (1968) Clin. Chim. Acta 22, 393-397.
6. Zak, B., Moss, N., Boyle, A.J., and Zlatkis, A. (1954) Anal. Chem. 26, 776-777.
7. Bartlett, G.R. (1959) J. Biol. Chem. 234, 466-468.
8. Glock, G.E., and McLean, P. (1953) Biochem J. 55, 400-408.
9. Ochoa, S. (1955) Methods Enzymol. 1, 739-753.
10. Nakanishi, S., and Numa, S. (1970) Eur. J. Biochem. 16, 161-173.
11. Brandes, R., Olley, J., and Shapiro, B. (1963) Biochem. J. 65, 244-247.
12. Iritani, N., and Fukuda, E. (1980) J. Nutr. 110, 1138-1143.
13. Weiss, S.B., Kennedy, E.P., and Kiyasu, J.Y. (1960) J. Biol. Chem. 235, 40-45.
14. Podack, E.R., Lakomek, M., Saathofe, G., and Seubert, W. (1974) Eur. J. Biochem. 45, 1-23.
15. Mercuri, O., Peluffo, R.O., and Thomas, M.E. (1974) Biochim. Biophys. Acta 369, 264-268.
16. Tanabe, T., Nakanishi, S., Hashimoto, T., Ogiwara, H., Nikawa, J., and Numa, S. (1981) Methods Enzymol. 71, 5-16.
17. Song, C.S., and Kim, K.H. (1981) J. Biol. Chem. 256, 7786-7788.
18. Matsuda, T., and Yugari, Y. (1967) J. Biochem. 61, 535-540.
19. Rutter, W.J., and Lardy, H.A. (1958) J. Biol. Chem. 233, 374-382.
20. Yeung, K.K., and Carrico, R.J. (1976) Anal. Biochem. 74, 369-375.
21. Iritani, N., Fukuda, E., Inoguchi, K., Tsubosaka, M., and Tashiro, S. (1980) J. Nutr. 110, 1664-1670.
22. Ouchterlony, O. (1958) Prog. Allergy 5, 1-78.
23. Iritani, N., Nakanishi, S., and Numa, S. (1969) Life Sci. 8, 1157-1165.
24. Iritani, N., Yamashita, S., and Numa, S. (1976) J. Biochem. 80, 217-222.
25. Iritani, N., and Fujikawa, S. (1982) J. Nutr. Sci. Vitaminol. 28, 621-629.
26. Castell, J.D., Sinnhuber, R.O., Wales, J.H., and Lee, D.J. (1972) J. Nutr. 102, 77-86.
27. Castell, J.D. Sinnhuber, R.O., Lee, E.J., and Wales, J.H. (1972) J. Nutr. 102, 87-92.
28. Castell, J.D., Lee, D.J., and Sinnhuber, R.O. (1972) J. Nutr. 102, 93-100.
29. Silpananta, P., and Goodridge, A.G. (1971) J. Biol. Chem. 246, 5754-5761.
30. Numa, S., Ringelmann, E., and Riedel, B. (1966) Biochem. Biophys. Res. Commun. 24, 750-757.
31. Numa, S., Goto, T., Ringelmann, E., and Riedel, B. (1967) Eur. J. Biochem. 3, 124-128.
32. Mackall, J., and Lane, M.D. (1977) Biochem. J. 162, 635-642.
33. Kaul, L., and Berdanier, C.D. (1975) J. Nutr. 105, 1132-1140.
34. Iritani, N., Fukuda, H., and Fukuda, E. (1981) Biochim. Biophys. Acta 665, 636-639.

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Urinary Malondialdehyde as an Indicator of Lipid Peroxidation in the Diet and in the Tissues

H.H. DRAPER,* L. POLENSEK,[1] M. HADLEY and L.G. MCGIRR, *Department of Nutrition, College of Biological Science, University of Guelph, Guelph, Ontario, Canada N1G 2W1*

ABSTRACT

Although malondialdehyde (MDA) is extensively metabolized to CO_2, small amounts are nevertheless excreted in an acid-hydrolyzable form in rat urine. In this study, urinary MDA was evaluated as an indicator of lipid peroxidation in the diet and in the tissues. MDA was released from its bound form(s) in urine by acid treatment and determined as the TBA-MA derivative by HPLC. MDA excretion by the rat was found to be responsive to oral administration of the Na enol salt and to peroxidation of dietary lipids. Urinary MDA also increased in response to the increased lipid peroxidation in vivo produced by vitamin E deficiency and by administration of iron nitrilotriacetate. Chronic feeding of a diet containing cod liver oil led to increases in MDA excretion which were not completely eliminated by fasting or feeding a peroxide-free diet, indicating that there was increased lipid peroxidation in vivo. MDA excretion was not responsive to Se deficiency or CCl_4 administration. DPPD, a biologically active antioxidant, but not BHA, a non-biologically active antioxidant, prevented the increase in MDA excretion in vitamin E deficient animals. The results indicate that MDA excretion can serve as an indicator of the extent of lipid peroxidation in the diet and, under conditions which preclude a dietary effect, as an index of lipid peroxidation in vivo.
Lipids 19:836-843, 1984.

INTRODUCTION

Current methods of determining the effect of prooxidants, antioxidants, drugs and other agents on lipid peroxidation in vivo consist of monitoring the exhalation of gaseous hydrocarbon residues of peroxidized fatty acids or analyzing the tissues for products of peroxidized lipids such as malondialdehyde (MDA), conjugated dienes or "lipofuscin" pigments (1). The first method requires specialized equipment for the separation, collection and measurement of respiratory gases uncontaminated by gases of bacterial origin. The second requires invasive or terminal procedures.

In a study on the metabolism of [1,3-^{14}C] MDA in the rat, Siu and Draper (2) observed that about 10% of the radioactivity administered orally was excreted in the urine. Analysis of rat urine revealed the presence of MDA in an acid-hydrolyzable form. These observations suggested that the urinary excretion of MDA might serve as an indicator of MDA consumed in the diet and/or formed in the tissues.

In the present study, urinary MDA was found to be responsive to MDA intake as well as to conditions associated with increased lipid peroxidation in vivo: vitamin E deficiency, iron administration and a high tissue concentration of polyunsaturated fatty acids (PUFA).

*To whom correspondence should be addressed.

[1] Part of this research was performed in fulfillment of the requirements for the MSc degree in nutrition.

METHODS

Experiment 1

The enolic sodium salt of MDA (Na MDA) was prepared from tetramethoxypropane and purified as described previously (3). It was administered to Wistar rats weighing ~330 g by stomach intubation in 1 ml 0.9% NaCl solution at a dose of 2 μg per g body weight. Urine and feces were collected at 12-hr intervals for 48 hr and frozen. MDA in the feces and diet (Purina Laboratory Chow) was determined by the HPLC procedure of Bird et al. (4) for food and tissues. Urinary MDA was determined by the following modification of this method. One ml of urine (and cage washings) and 1 ml of saturated thiobarbituric acid (TBA) solution were placed in a screw-cap culture tube and the pH was adjusted to 3.0 ± 0.1 with HCl (4N,1N, 0.1N). The tubes were capped, heated in a boiling water bath for 30 min and cooled to room temperature. The sample was applied to a Sep-pak™ C_{18} cartridge (Waters) after pretreating the cartridge with 10 ml methanol (HPLC grade), 15 ml double-distilled water and flushing with air. MDA was eluted using 4 ml methanol and the eluate was dried at 70 C on a sand bath under a stream of air. The residue was dissolved in 2 ml double-distilled water and an aliquot (10-200 μl) was injected onto the column (4) (Fig. 1).

Urine was collected under toluene and stored in the frozen state. Urine MDA values

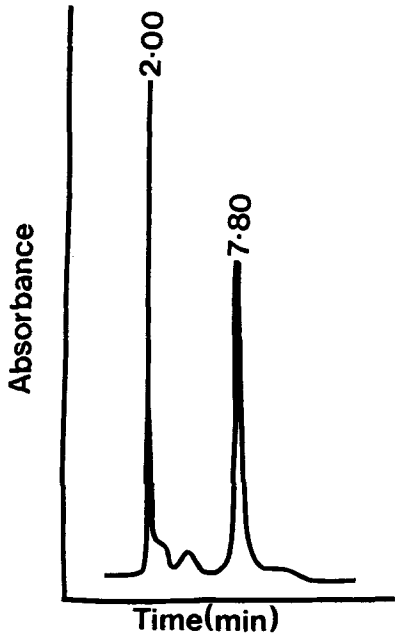

FIG. 1. HPLC elution profile for the TBA derivative of MDA. 0.39 × 30 cm μBondapak C_{18} stainless steel column attached to a 3 × 22 cm guard column packed with Bondapak C_{18}/Corasil (Waters). 546 nm interference filter attached to an electronic integrator. Mobile phase 17% methanol. For details see text and Bird et al. (4). MDA at 7.80 min.

were not affected by standing at room temperature for 24 hr or by prolonged storage of frozen samples. Chromatographic analysis demonstrated that there is little, if any, free MDA in rat urine; MDA determined by the TBA procedure is released from "bound forms" by acid hydrolysis (L. G. McGirr, M. Hadley and H. H. Draper, unpublished results).

Experiment 2

The effect of feeding a high PUFA diet on MDA excretion in the urine was determined. MDA was measured in 24-hr urine samples obtained from animals fed diets high or low in PUFA for 9 weeks. To assess the endogenous contribution to urinary MDA, the animals then were fed a diet devoid of PUFA for 48 hr, and urinary MDA was determined during the second 24 hr period. Finally, the animals were fasted for 48 hr and MDA excretion during the final 24 hr was measured. Liver and epididymal adipose tissue samples were taken for fatty acid analysis.

The composition of the diets (%) was as follows: cornstarch 32.76; glucose 30.60; cellulose 2.50; "vitamin-free" casein 15.00; fat 15.00; $CaCO_3$ 1.03; $Ca(H_2PO_4)_2 \cdot H_2O$ 0.86; $MgCO_3$ 0.69; choline chloride 0.10; DL-methionine 0.05; vitamin mix (5) 0.50; mineral mix (5) 0.91. The fat portion of the diets consisted of 10% molecular-distilled ("stripped") corn oil plus 5% cod liver oil (high PUFA), 10% hydrogenated coconut oil plus 5% stripped corn oil (low PUFA) or 15% hydrogenated coconut oil (no PUFA).

Experiment 3

Two groups of 7 male weanling Wistar rats were fed vitamin E deficient diets for 28 weeks. One group was fed a basal diet containing 10% stripped corn oil and the other a similar diet containing 10% stripped lard. Control groups were fed the same diets supplemented with 30 IU of vitamin E per kg as DL-α-tocopheryl acetate. The composition of the diets was similar to that described for Experiment 2 except that the fat content was 10% and the carbohydrate consisted of 68.26% glucose. Vitamin E depletion was monitored by the autohemolysis test (6) and by depression of weight gain.

Experiment 4

The effect on urinary MDA of administering ferric nitrilotriacetate (Fe NTA) was determined using rats fed the stripped corn oil basal diet for 10 wk with and without vitamin E (30 IU per g). Fe NTA was prepared by combining 1.5 volumes of a 0.1M solution of nitrilotriacetate (NTA) in H_2O with 1 volume of 0.1M $Fe(NO_3)_3 \cdot 9H_2O$ in 0.4N HCl and adjusting the pH to 7.2 using solid $NaHCO_3$ (G. Goddard, personal communication). Fresh Fe NTA solution was injected intraperitoneally in amounts providing either 3 mg or 9 mg Fe per kg body wt. Controls were given equivalent amounts of NTA.

Experiment 5

The effect on MDA excretion of feeding the necrogenic selenium and vitamin E deficient Torula yeast diet of Schwartz (7) was investigated. Three groups of 7 weanling rats were fed the basal diet alone, supplemented with vitamin E (30 IU per kg) or supplemented with vitamin E and selenium (0.2 ppm as Na_2SeO_3). Erythrocyte hemolysis, plasma Se and urinary MA were monitored for 6 weeks. Selenium was determined by a modification of the fluorometric method of Hoffman et al. (8).

Experiment 6

The effect of carbon tetrachloride (CCl_4) administration on MDA excretion was determined. Urine, plasma and liver were analyzed

for MDA by the HPLC method of Bird et al. (4) 24 hr after oral intubation of normal adult rats with 100 μl CCl_4 per 100 g. Additional animals were given repeated oral doses over 3 days (100, 50 and 50 μl per 100 g, respectively). The effect of vitamin E depletion (12 wk) and fasting (which has been reported to potentiate CCl_4 hepatoxicity) on the urinary MDA response to CCl_4 also was determined.

Experiment 7

MDA is formed as a product of the metabolism of endoperoxides in the cyclooxygenase reaction of prostaglandin synthesis, and cyclooxygenase products are formed in increased amounts in vitamin E deficient rats (Machlin, 1978; Hwang and Donovan, 1982). This increase is inhibited by administration of aspirin, a cyclooxygenase inhibitor. Hence the effect of aspirin on MDA excretion was investigated as a means of differentiating between the effects of vitamin E deficiency on the excretion of MDA formed in the course of prostaglandin metabolism and MDA formed as a result of nonenzymatic lipid peroxidation in vivo. Weanling rats were fed the vitamin E deficient stripped corn oil diet until they exhibited a positive hemolysis test and increased MDA excretion, then were given aspirin mixed into the diet at either 0.3% or 0.6%. MDA was determined 3 days later on 24 hr urine samples. The effect of aspirin also was tested on controls given 100 IU vitamin E/g diet.

RESULTS

Na MDA Administration

Stomach intubation with Na MDA at a level of 2 μg/g body weight elicited significant increases in MDA in the feces and urine by the TBA-HPLC procedure (Table 1). Chromatography of the urine on an ion exchange column demonstrated that none of the MDA was present in the free form (unpublished results).

PUFA Intake

Table 2 illustrates the effect of chronic PUFA and vitamin E intake on urinary MDA excretion in fed and fasted rats. MDA excretion was markedly increased by feeding the high

TABLE 1

Effect of Intubating Adult Rats with the Na enol Salt of MDA on MDA Excretion in the Urine and Feces

Treatment	MDA intake (μg)		MDA excretion (μg/48 hr)	
	Dose	Diet	Feces	Urine
Control - stock diet	–	287	80 ± 7[b]	47 ± 7
Na MDA - 2 μg/g BW[a]	652	264	108 ± 18[c]	93 ± 10[c]

[a]Body weight.
[b]$\bar{x}$ ± SEM.
[c]$P < 0.05$, n = 5.

TABLE 2

Effect of PUFA and Vitamin E Intake on MDA Excretion in the Urine

Diet sequence	Time	Vitamin E (IU/g diet)[a]		
		0	30	100
High PUFA	9 wk	10.7 ± 0.9[b]	10.7 ± 1.8	8.1 ± 0.8
No PUFA	48 hr[c]	2.7 ± 0.2	1.7 ± 0.1	1.3 ± 0.06
Fast	48 hr[c]	4.8 + 1.6	2.9 ± 0.2	2.3 ± 0.3
Low PUFA	9 wk	2.7 ± 0.3	1.1 ± 0.1	1.0 ± 0.1
No PUFA	48 hr[c]	1.6 ± 0.1	0.8 ± 0.04	0.6 ± 0.02
Fast	48 hr[c]	1.9 ± 0.2	1.3 ± 0.2	1.1 ± 0.1

[a]All-rac-α-tocopheryl acetate.
[b]μg MDA/24 hr, $\bar{x}$ ± SEM, N = 8.
[c]MDA values are for the last 24 hr of the 48-hr period.
$P < 0.01$ for effects of PUFA, vitamin E and fasting.

PUFA diet. Swtiching the animals to a PUFA-free diet resulted in a major decrease in MDA excretion by the high PUFA group, indicating that most of the urinary MDA was of dietary origin. Analysis of diets formulated to contain different mixtures of the fats and oils used in this experiment showed that most of the MDA in the high PUFA diet was formed from the cod liver oil component. Use of the acetate form of vitamin E had a permissive effect on fatty acid peroxidation in the diet.

Feeding a PUFA-free diet reduced, but did not eliminate, the difference in MDA excretion between animals fed high vs. low PUFA diets (Table 2). To preclude a possible effect on MDA excretion of any residues of the original diets remaining in the gut or recycled by coprophagy, the animals were fasted for 48 hr after being fed the PUFA-free diet, and urine collected during the final 24 hr of the fast was analyzed for MDA. Rather than a further decrease, fasting resulted in a small but significant increase in MDA excretion in both groups (Table 2). The approximately two-fold difference in MDA excretion between groups previously fed high vs. low PUFA diets persisted, indicating that it was due to a difference in MDA formation in vivo.

There was a significant effect of vitamin E intake on MDA excretion except in the animals consuming the high PUFA diet, in which case urinary MDA was derived predominantly from the diet (Table 2). Although MDA excretion tended to be lower at the 100 IU/g level of vitamin E intake than at 30 IU/g, none of the differences was significant ($P > 0.05$). Suppression of MDA excretion during consumption of the PUFA-free diet and during fasting indicates that the effect of the vitamin on urinary MDA was due to inhibition of lipid peroxidation in the tissues. It is noteworthy, however, that even at a high vitamin E intake (100 IU/g diet), MDA excretion from endogenous sources was twice as great in the animals previously fed the high PUFA diet.

Analysis of liver and epididymal fat pads confirmed the enrichment of the tissues of the rats fed the high PUFA diet with cod liver oil fatty acids (Table 3).

Vitamin E Deficiency

The effect of feeding the vitamin E deficient corn oil diet on urinary MDA is illustrated in Figure 2. A difference in MDA excretion between deficient animals and controls was discernible after 6 weeks, and by 24 weeks there was a 5-fold differential. Supplementing the deficient diet with vitamin E (100 IU/kg) resulted in a fall in MDA excretion to the control level. Increased MDA excretion was associated with a significant increase in red blood cell hemolysis at 9 weeks ($P < 0.05$).

On a body weight basis, MDA excretion declined during growth in the +E rats and then

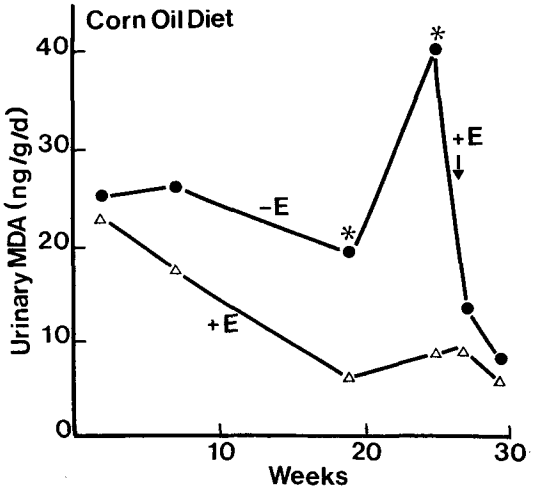

FIG. 2. Excretion of MDA in the urine of rats fed the stripped corn oil diet with and without vitamin E, expressed on a body weight basis. N = 7, *P < 0.05.

TABLE 3

Fatty Acid Composition of Liver and Adipose Tissue of Rats Fed High and Low PUFA Diets (%, w/w)

Diet	Saturated	Unsaturated	18:2	18:3	20:4	20:5	22:6
			Liver				
High PUFA	37.6 ± 0.9[a]	62.4 ± 0.9	22.6 ± 0.5	1.4 ± 0.1	7.9 ± 0.5	1.6 ± 0.2	5.7 ± 0.6
Low PUFA	45.3 ± 0.9	54.7 ± 0.9	15.2 ± 0.6	0.2 ± 0.1	15.8 ± 0.6	0.1 ± 0.1	1.2 ± 0.2
			Epididymal fat pad				
High PUFA	28.2 ± 0.6	71.8 ± 0.6	30.3 ± 0.7	2.8 ± 0.1	0.4 ± 0.1	<0.2	n.d.
Low PUFA	39.3 ± 1.0	60.7 ± 1.0	23.4 ± 0.6	n.d.	2.4 ± 0.9	<0.03	n.d.

[a]Mean ± SEM, N = 8.

n.d. = none detected.

stabilized (Fig. 2). Total excretion increased from 1.7 μg/d after 1 wk to 3.6-5.5 μg/d from 7 to 25 weeks. In the −E animals, MDA excretion increased from 1.8 μg/d after 1 wk to 16.3 μg/d at 25 weeks.

MDA excretion by the rats fed the lard diets is illustrated in Figure 3. In accordance with its lower PUFA content, the vitamin E deficient stripped lard diet produced a smaller increase in MDA excretion than the deficient corn oil diet. Nevertheless, a significant increase in urinary MDA ($P < 0.05$) was observed by 19 weeks. MDA excretion fell to control levels after vitamin E supplementation. Significant differences ($P < 0.05$) in hemolysis and body weight were recorded at 12 weeks and 19 weeks, respectively.

Iron Administration

The effect of Fe NTA administration on MDA excretion is shown in Table 4. In the vitamin replete rats, there was a 2.4-fold increase in urinary MDA during the 24-hr period following a dose of 3 mg Fe/kg and a 5-fold increase following a dose of 9 mg Fe/kg. Fe NTA at the lower dose was fatal to all 8 vitamin E deficient rats.

Selenium/Vitamin E Depletion

The selenium/vitamin E deficient Torula yeast diet produced no increase in erythrocyte hemolysis or MDA excretion (Table 5). After 4 weeks, mortality from liver necrosis increased rapidly in the unsupplemented group until at 6 weeks only 2 animals remained. No difference in MDA excretion was found in the 5-week survivors. Plasma selenium was markedly lower in the rats unsupplemented with this element.

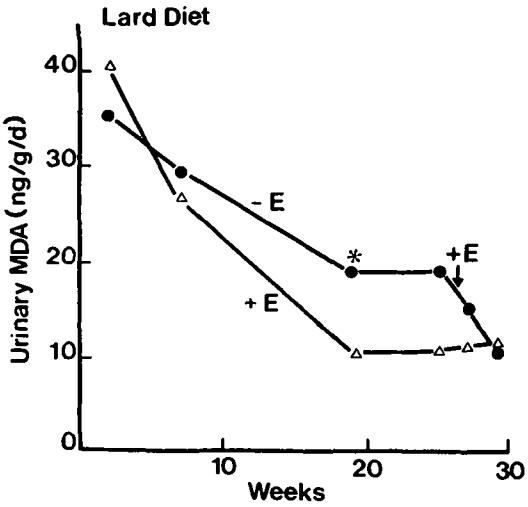

FIG. 3. Urinary excretion of MDA by rats fed the stripped lard diet with and without vitamin E. N = 7, *$P < 0.05$.

TABLE 4

Stimulation of Urinary MDA Excretion in the Rat Produced by Intraperitoneal Administration of Ferric Nitrilotriacetate (Fe NTA)

Treatment	Urinary MDA μg/24 hr
+E	1.16 ± 0.22[a]
−E	3.84 ± 0.72[c]
+E + NTA	1.09 ± 0.12
+E + Fe NTA (3 mg Fe/kg)	2.63 ± 0.34[c]
+E + NTA	1.44 ± 0.15
+E + Fe NTA (9 mg Fe/kg)	7.41 ± 0.75[c]
−E + NTA	3.84 ± 0.68
−E + Fe NTA (3 mg Fe/kg)	–[b]

[a] $\bar{x}$ ± SEM.
[b] All died.
[c] $P < 0.01$, N = 8.

CCl_4 Administration

No consistent effect of CCl_4 administration on MDA excretion was discernible (data not shown). Analysis of plasma and liver also revealed no differences in MDA content attributable to CCl_4 treatment.

Fasting

Since urinary MDA is responsive to oral MDA administration, the possibility that the increased MDA excretion by the vitamin E deficient animals was due to a greater intake of MDA generated in the antioxidant deficient basal diets during storage or feeding was tested by comparing the MDA excretion of fasted deficient and control rats. The increased MDA excretion by the vitamin E deficient rats fed the corn oil diet was unaffected by fasting (Fig. 4), indicating that the increase was of endogenous origin. There also was no increase in MDA excretion during fasting in rats fed the 10% corn oil diet, in contrast to those fed a diet containing 5% cod liver oil in addition to 10% corn oil (Table 2). This difference in response may have been due to the shorter period of fasting imposed on the corn oil animals. Alternatively, it may indicate that the increase in MDA excretion observed in Experiment 2 was caused by peroxidation of highly unsaturated fatty acids of the ω-3 series released by fasting lipolysis.

MDA excretion by the control rats fed the stripped lard diet was consistently higher than that by the controls fed the stripped corn oil

TABLE 5

Effect of Feeding Torula Yeast Diet on Plasma Se, Hemolysis and Urinary MDA[a]

Diet	Plasma Se ppm	% Hemolysis	Urinary MDA μg/24 hr
+E +Se	0.37 ± 0.03	2.6 ± 0.4	3.77 ± 0.52
+E −Se	0.09 ± 0.02[b]	2.9 ± 0.4	3.92 ± 0.46
−E −Se	0.06 ± 0.01[b]	4.5 ± 0.8	4.03 ± 0.44

[a]After 4 seeks, N = 6, $\bar{x}$ ± SEM.
[b]$P < 0.01$.

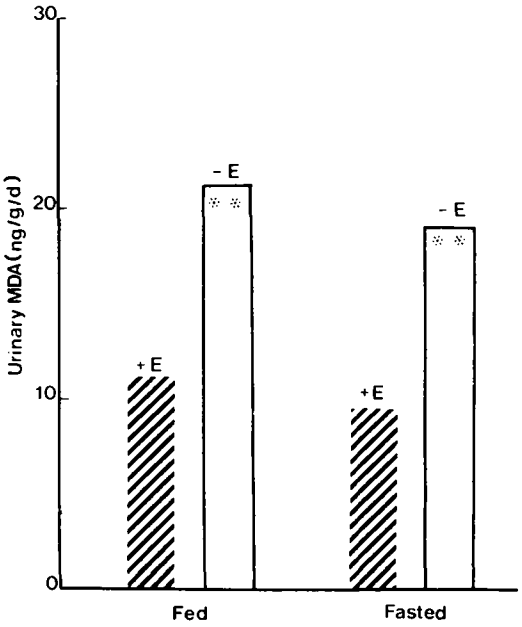

FIG. 4. Effect of fasting on MDA excretion by rats fed the stripped corn oil diet (**P < 0.01).

diet (Fig. 2 and 3). There was no difference in the MDA content of the frozen diets (0.3-0.4 μg/g), but an increase in the MDA content of the stripped lard diet to 1.24 μg/g was observed when it was kept at room temperature for 48 hr. No increase was seen in the stripped corn oil diet under the same conditions. Feeding the vitamin E deficient lard diet, but not the deficient corn oil diet, led to an immediate increase in MDA excretion in the urine. Fasting eliminated the difference in MDA excretion between animals fed the 2 diets. Hence, the difference in MDA excretion observed between vitamin E replete rats fed the lard and corn oil diets was due to ingestion of MDA formed from fatty acids in the stripped lard during the feeding procedure. No increase in MDA content was observed over 48 hr at room temperature in the diet in which stripped lard was replaced with commercial lard. These observations indicate that peroxidized arachidonic acid was the primary source of MDA formed in the stripped lard diet.

BHA and DPPD

Since the food antioxidant butylated hydroxyanisole (BHA) is ineffective as a substitute for vitamin E in vivo, it was of interest to compare its effects on the elevated MDA excretion of vitamin E deficient animals with those of the biologically active antioxidant N,N-diphenyl-p-phenylenediamine (DPPD) (11). After 1 wk of BHA administration to deficient rats at a level of 0.1% of the diet, fasting MDA excretion was not significantly reduced (3.9 μg/d vs. 3.5 μg/d). After 1 wk of 0.1% DPPD feeding MDA excretion had decreased to 2.3 μg/d ($P < 0.01$).

Aspirin

Aspirin administration had no effect on MDA excretion in vitamin E-replete animals, but the increase due to vitamin E deficiency was modulated significantly ($P < 0.05$) (Fig. 5). Similar results were obtained by feeding 0.3% and 0.6% aspirin. The data indicate that vitamin E deficiency results in some increase in the excretion of MDA arising from prostaglandin metabolism, but that most of the increase in

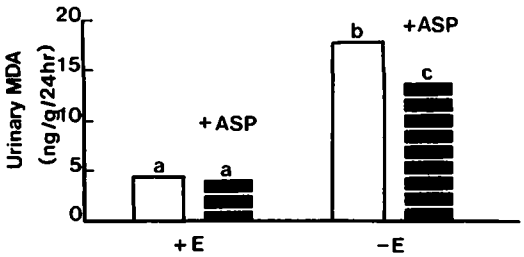

FIG. 5. Effect of feeding 0.6% aspirin (ASP) on MDA excretion by vitamin E deficient and control rats. Values with different superscripts are significantly different ($P < 0.05$).

MDA excretion is due to cyclooxygenase-independent processes.

DISCUSSION

This study shows that urinary MDA is responsive to lipid peroxidation in the diet and that, under conditions in which the influence of peroxidized dietary lipids is precluded, MDA excretion reflects the rate of lipid peroxidation in vivo. Responsiveness to increased in vivo peroxidation was seen in the examples of peroxidation caused by iron loading, vitamin E deficiency and a high PUFA intake.

The marked response in urinary MDA to parenteral administration of Fe NTA is consistent with the increase in pentane evolution produced by this compound in mice (12) and by iron dextran in rats (13). The vulnerability of vitamin E deficient animals to iron-catalyzed lipid peroxidation is evident from the uniformly lethal effect of Fe NTA on deficient animals at a dose which had no gross effects on the controls.

Although vitamin E deficiency produced an increase in MDA excretion, urinary MDA in replete animals decreased with maturation on a body weight basis (Fig. 2). This observation, coupled with the increase in MDA excretion in cod liver oil-fed rats during fasting (Table 2), suggests that MDA formation in vivo may be increased under conditions of enhanced turnover of tissue lipids.

A specificity for vitamin E or another biologically active lipid antioxidant for the modulation of MDA formation in vivo is indicated by the effectiveness of DPPD, and the ineffectiveness of BHA, in reducing the elevated MDA excretion of vitamin E deficient animals. A similar disparity in activity between these antioxidants has been reported with respect to the suppression of pentane evolution in iron-loaded rats (13). Although BHA is an effective food antioxidant, it is inactive as a substitute for vitamin E in the prevention and treatment of vitamin E deficiency diseases in animals (11). These observations provide further evidence that the biological role of vitamin E is associated with its antioxidant properties.

Failure to observe an increase in urinary MDA in animals fed the selenium/vitamin E deficient Torula yeast diet indicates that MDA formation in vivo is mainly a vitamin E-dependent process. Although their selenium status was low (Table 2), the deficient animals were only moderately depleted of vitamin E, as indicated by negative hemolysis tests and the longer depletion periods required to produce an increase in MDA excretion by rats fed a diet deficient in vitamin E alone (Fig. 2 and 3). Hill and Burk (14) observed that, whereas hepatocytes isolated from vitamin E-deficient rats contained increased amounts of TBA-reactive substances, there was no increase in cells derived from selenium-deficient animals. The failure of MDA to accumulate in selenium deficiency may be explained by the fact that the primary substrate for selenium-dependent glutathione peroxidase is H_2O_2 rather than fatty acyl peroxides (15). Selenium is ineffective in the prevention of most diseases caused by feeding diets lacking vitamin E or another biologically active lipid antioxidant.

The failure of CCl_4 administration to cause an increase in MDA excretion is surprising in view of its capacity to increase pentane evolution (1,13). However, while some investigators have reported evidence of lipid peroxidation in the liver following CCl_4 administration, several groups have failed to find increases in TBA-reactive substances, diene conjugation or MDA-derived fluorescent products. De Toranzo and Castro (16) concluded that MDA-derived fluorescent products are not formed in rat liver during CCl_4 intoxication, and Harris et al. (17) found no increase in diene conjugation even though pentane exhalation was enhanced. The present study supports the view that CCl_4 does not increase MDA formation in the tissues. It is possible that lipid dienyl radicals generated in the liver, via an attack on lipids by the $\cdot CCl_3$ radical (18) decompose to form alkanes but do not undergo significant oxidative decomposition to form MDA.

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REFERENCES

1. Tappel, A.L. (1980) in Free Radicals in Biology (Pryor, W.A., ed.) Vol. IV, pp. 1-47, Academic Press, New York.
2. Siu, G.M., and Draper, H.H. (1982) Lipids 17, 349-355.
3. Siu, G.M., and Draper, H.H. (1983) J. Toxicol. Envir. Health 11, 105-119.
4. Bird, R.P., Hung, S.S.O., Hadley, M., and Draper, H.H. (1983) Anal. Biochem. 128, 240-244.
5. Siu, G.M., Hadley, M., and Draper, H.H. (1981) J. Nutrition 111, 1681-1685.
6. Draper, H.H., and Csallany, A.S. (1969) J. Nutrition 98, 390-394.
7. Schwartz, K. (1954) Ann. New York Acad. Sci. 57, 878-888.
8. Hoffman, T., Westerby, R.J., and Hidiroglou, M. (1968) J. Am. Oil Chem. Soc. 51, 1039-1042.
9. Machlin, L.J. (1978) in Tocopherol, Oxygen and Biomembranes (de Duve, C., and Hayaishi, O.,

eds.) pp. 179-190, Elsevier, Amsterdam.
10. Hwang, D.H., and Donovan, J. (1982) J. Nutrition 112, 1233-1237.
11. Draper, H.H. (1980) in Vitamin E: A Comprehensive Treatise (Machlin, L.J., ed.) pp. 272-288, Marcel Dekker, Inc., New York.
12. Goddard, G., and Sweeney, G. (1983) Proc. Can. Fed. Biol. Soc. 23, 237.
13. Dillard, C.J., Downey, J.E., and Tappel, A.L. (1984) Lipids 19, 127-133.
14. Hill, K.E., and Burk, R.F. (1982) J. Biol. Chem. 257, 10668-10672.
15. McCay, P.B., Gibson, D.D., Fong, K-L., and Hornbrook, K.R. (1976) Biochim. Biophys. Acta 431, 459-468.
16. de Toranzo, E.G.D., and Castro, J.A. (1980) Res. Comm. Chem. Path. Pharmacol. 29, 385-388.
17. Harris, R.N., Ratnayake, J.H., Garry, V.F., and Anders, M.W. (1982) Toxicol. Appl. Pharmacol. 63, 281-291.
18. McCay, P.B., Lai, E.K., Poyer, J.L., DuBose, C.M., and Janzen, E.G. (1984). J. Biol. Chem. 259, 2135-2143.

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The Influence of Protein and Carbohydrate Type on Serum and Liver Lipids and Lipoprotein Cholesterol in Rabbits

JOHN E. BAUER* and **STEVEN J. COVERT**, *Department of Physiological Sciences, College of Veterinary Medicine, University of Florida, Gainesville, FL 32610*

ABSTRACT

The non-lipid portions of semi-synthetic diets appear to be important determinants of hypercholesterolemia and atherosclerosis in the rabbit. Serum and liver lipid concentrations were determined in rabbits which had been pair-fed various protein (casein or soy protein isolate) and carbohydrate (sucrose or dextrose) sources as part of low fat, low cholesterol, semi-synthetic diets. It was verified that casein-containing diets render rabbits hypercholesterolemic, while soy protein caused a degree of hypocholesterolemia. Additionally, sucrose, when fed in conjunction with casein, appears to augment this hypercholesterolemic effect. The distribution of total cholesterol among lipoprotein subclasses was increased in both the intermediate density lipoprotein (IDL) (1.006-1.019 g/ml) and low density lipoprotein (LDL) (1.019-1.063 g/ml) fractions and decreased in the high density lipoprotein (HDL) (1.063-1.21 g/ml) fraction when casein is fed. Soy protein feeding caused relatively more cholesterol to appear only in the IDL fraction when compared with commercial chow fed rabbits. Reasons for these differences may involve the saturation or suppression of endogenous lipoprotein hepatic receptors.
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INTRODUCTION

Hypercholesterolemia and atherosclerosis have been produced in rabbits by feeding cholesterol-free, semi-purified diets containing animal protein (1,2). The replacement of this protein type by soy protein isolate has been shown to lower plasma cholesterol concentrations in both normocholesterolemic and hypercholesterolemic human subjects (3) as well as in laboratory animals (4,5). The non-lipid portion of the diet, therefore, appears to be important in the development of hypercholesterolemia and atherosclerosis seen in rabbits when fed low fat, low cholesterol, semi-synthetic diets (6). However, the types and amounts of fat used in many of these previous studies may have been an additional factor in the biochemical changes observed in these animals (7,8).

The objective of this study was to determine the effects of various protein and carbohydrate sources when fed as part of a low fat, low cholesterol, semi-synthetic diet on serum and liver lipid concentrations in the rabbit. Free and esterified cholesterol and phospholipid content was measured at the end of the experiment. In addition, the distribution of cholesterol in the different serum lipoprotein fractions of these rabbits is reported.

MATERIALS AND METHODS

Animals and Experimental Design

In the experiment, 30 3-month old, New Zealand white rabbits, matched for size (ca. 2 kg), were used. The animals were housed individually in stainless steel cages in a room with controlled lighting (12 hr) and constant temperature and humidity. On arrival at the animal facility, the rabbits were maintained on commercial rabbit pellets (Ralston Purina Co., St. Louis, Missouri) for 2 weeks to accustom them to their new environment.

A group consisting of 6 rabbits continued to receive the commercial diet during the experimental period of 84 days. The other animals were transferred at this time to one of 4 semi-purified diets (ICN Nutritional Biochemicals, Cleveland, Ohio) the general composition of which is indicated in Table 1. Protein sources included either casein or soy protein isolate while carbohydrate was supplied by either dextrose or sucrose. The rabbits (6 animals per group) were pair-fed the pelleted diets containing either casein-sucrose (CS) and soy protein-sucrose (SS) or casein-dextrose (CD) and soy protein-dextrose (SD) during the course of the experiment.

Analytical Methods

During the feeding period, the animals were weighed every 2 weeks. Blood samples were taken from a marginal ear vein on days 17, 52 and 84 without prior fasting between 9 and 10:30 a.m. and allowed to clot at room temperature. The serum was isolated after low speed centrifugation. Serum total cholesterol and triglyceride concentrations were estimated enzymatically, on the day of collection, using

*To whom correspondence should be addressed.

TABLE 1

Composition of Diets (g/100 g feed)

Protein[a]	25.0
Carbohydrate	51.5
Corn oil	2.5
USP XIV salt mixture	5.0
Cellulose (alphacel)	15.0
Vitamin pre-mix[b]	1.0

[a]In the case of soy protein-isolate diets, 1.0 mg d, l methionine/lb feed was added.

[b]Amounts in g/100 lbs diet: vitamin A (200,000 units/g), 4.5; vitamin D (400,000 units/g), 0.25; α-tocopherol acetate, 5.0; ascorbic acid, 45.0; inositol, 5.0; choline chloride, 75.0; menadione, 2.25; p-amino benzoic acid, 5.0; niacin, 4.5; riboflavin, 1.0; pyridoxine hydrochloride, 1.0; thiamine hydrochloride, 1.0; calcium pantothenate, 3.0; in Mg/100 lbs diet: biotin, 20; folic acid, 90; vitamin B_{12}, 1.35.

kits supplied by Gilford Diagnostics (Gilford Instrument Laboratories, Cleveland, Ohio).

At the end of the experimental period, animals were killed by exsanguination. Livers were rapidly excised and stored at -70 C until the time of analysis. Known amounts of liver tissue were homogenized in $CHCl_3$:MeOH (2:1 v/v) in an Omni-Mixer (DuPont Instruments, Newton, Connecticut) and total lipid extracts prepared according to Folch et al. (9). Serum samples collected at the same time were similarly extracted. Liver total cholesterol in the extracts was determined by a fluorometric technique based on the Lieberman-Burchard reaction (10). The lipid phosphorus concentrations were determined as described by Bartlett (11). Free and esterified cholesterol of both liver and serum extracts were estimated spectrophotofluorometrically after fractionation of the lipids by TLC in hexane:diethyl ether:glacial acetic acid (90:10:1 v/v/v) as previously described (12). The serum lipoprotein classes were separated with a Beckman Ti 50 rotor into 4 density classes by sequential density ultracentrifugation, according to Hatch and Lees (13). Total cholesterol in the fractions was determined enzymatically as for serum.

RESULTS

The mean body weights of the pair-fed rabbit groups are presented in Table 2. While pair feeding somewhat restricted these animals' intake, their body weights were maintained throughout the feeding period. It should be noted that the commercial chow fed rabbits gained considerably more weight during this same period of time. However, these animals were fed ad libitum. This disparity in body weight indicated that the rabbits used in this study, purchased as a single lot, were in a growth stage of life. This information is useful since it has been reported previously that growing rabbits are more susceptible to semi-synthetic diet induced hypercholesterolemia than mature animals (14). On the other hand, restricted feeding of semi-purified diets has been reported as preferable in these types of studies to best exclude possible interferences with the cholesterolemic response due to differences in growth rate between rabbits fed diets containing casein versus soy protein (15). Furthermore, the average caloric intake of the experimental diet fed rabbits approximated that generally regarded as necessary for maintenance but not growth (16), and is in agreement with the final weights observed in these animals.

Figures 1 and 2 show the serum cholesterol and triglyceride concentrations in the rabbits during the feeding period. Rabbits fed the CS diet rapidly became hypercholesterolemic, while those fed the CD diet became hypercholesterolemic more slowly. By contrast, rabbits fed either the SS or SD diets developed low normal serum cholesterol levels when compared with the chow fed group. Serum triglyceride levels in all groups were somewhat variable, with a statis-

TABLE 2

Body Weights of Rabbits Pair-Fed the Semi-Purified Diets[a]

	Body weight (grams)			
Dietary group[b]	Day 0	Day 17	Day 52	Day 84
CD	2560 ± 168	2533 ± 197	2652 ± 246	2877 ± 245
SD	2567 ± 173	2440 ± 231	2617 ± 316	2647 ± 328
CS	2778 ± 227	2671 ± 167	2882 ± 218	2881 ± 448
SS	2780 ± 233	2425 ± 220	2547 ± 228	2792 ± 280
C	2690 ± 418	3037 ± 470	3347 ± 533	3775 ± 636

[a]Values are expressed as mean ± S.D.

[b]Feed consumption averaged 55 g/day in the CS-CD pairs and 50 g/day in the SS-SD pairs. Rabbits on commercial chow diets were fed ad lib.

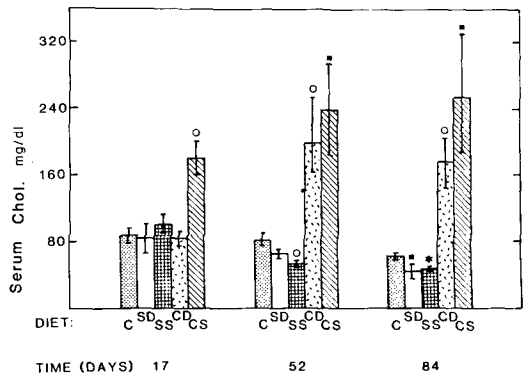

FIG. 1. Serum cholesterol concentrations of rabbits during the experimental period. For abbreviations of dietary groups, see text. Values are significantly different from chow fed animals at: * $p < 0.001$, o = $p < 0.01$, and ■ = $p < 0.05$ levels by the student t-test.

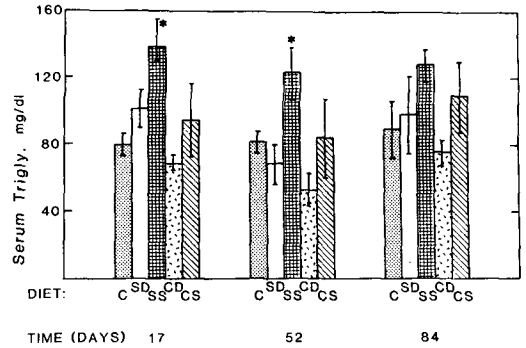

FIG. 2. Serum triglyceride concentrations of rabbits during the experimental period. For abbreviations of dietary groups, see text. Values are significantly different from chow fed animals at: * $p < 0.05$, levels by the student t-test.

tically significant increase in only the SS diet fed animals compared with the chow fed case.

Serum lipid concentrations among rabbits at the end of the dietary period can be found in Table 3. Rabbits fed the casein diets were hypercholesterolemic due to increases in both free and esterified cholesterol when compared with the soy protein diet fed animals. This effect was somewhat more pronounced when casein was fed in conjunction with sucrose, although it was not statistically significant. Some within-group variability was observed, especially in the CS diet fed animals, as evidenced by the large standard error. Along with the increased levels of both free and esterified cholesterol in the casein fed rabbits, the per cent free cholesterol and free to esterified cholesterol rarios in these groups also were elevated indicating that free cholesterol was increased more markedly relative

TABLE 3

Serum Lipid Concentrations in Rabbits Fed Different Diets for 84 Days (mg/dl ± S.E.)[a]

	Dietary group											
	CD		SD		CS		SS		C			
Total chol.	181	± 30[b,c]	48	± 9[c]	259	± 73[b,c]	43	± 4[b]	82	± 5[b,c]		
Free chol. (FC)	58	± 13[b]	11	± 2	90	± 38	8	± 1[b]	14	± 2		
Esterified chol. (EC)[g]	208	± 29[b,c]	63	± 13[b,c]	283	± 74[c,d]	59	± 5[b,d,e]	114	± 6[b,e]		
% FC	28	± 2[b,c]	23	± 5[d]	30	± 3[d,e,f]	18	± 3[b,e]	17	± 2[c,f]		
FC/CE	0.23	± 0.07[b,f]	0.18	± 0.05[d]	0.27	± 0.10[c,e]	0.14	± 0.06[e,f]	0.12	± 0.04[b,c,d]		
Triglyceride	77	± 8[b]	100	± 23	111	± 21	130	± 10[b]	91	± 17		

[a]Values bearing the same letter in a given row are significantly different ($P < 0.05$) by the student t-test. For dietary group abbreviations, see text.

[g]Esterified cholesterol determined as free cholesterol and corrected by the factor 1.67.

to its esterified form.

Autopsy data on the livers of the rabbits in this study is presented in Table 4. No appreciable differences among liver weights as per cent of body weight were found. In addition, no gross atherosclerotic lesions were seen in the aortas from any of the rabbits. Liver total cholesterol was elevated in the CD diet fed rabbits, but only when compared with the SD and CS groups. Free cholesterol tended to be decreased somewhat in the livers of the semi-purified diet fed animals when compared with the chow fed case except for the rabbits fed the SS diets. The liver esterified cholesterol concentrations were elevated in the casein fed rabbits when compared with either the soy protein or chow fed animals. Here, too, variability among rabbits fed the CS diet was observed. The percentage free cholesterol in livers of the casein fed rabbits was decreased when compared with either their soy protein pair-fed counterparts or the chow fed animals. Additionally, liver phospholipid content in livers of casein fed rabbits was decreased.

The distribution of cholesterol among lipoprotein classes of rabbits fed the various diets can be found in Table 5. The excess cholesterol in the casein fed animals was shown to be present mainly in the IDL (1.006-1.019 g/ml) and LDL (1.109-1.063 g/ml) fractions. Rabbits fed the soy protein diets showed only a small increase in their IDL cholesterol distribution. Serum HDL (1.063-1.21 g/ml) percentage cholesterol was found to be decreased in all semi-purified diet fed animals when compared with the chow fed rabbits. This change was most marked in the casein dietary groups. On an absolute basis, however, serum HDL cholesterol concentrations in the casein fed groups were elevated most likely because of the overall hypercholesterolemia observed in these rabbits. Calculated HDL/LDL ratios indicate decreased values for both casein diet fed groups, while the soy-protein fed rabbits were observed to have ratios similar to the chow fed case.

TABLE 4

Liver Autopsy Data on Rabbits Fed the Different Diets for 84 Days (mean ± S.E.)[a]

	Dietary Group				
	CD	SD	CS	SS	C
Liver weight (g)	81 ± 7	67 ± 4	77 ± 6	69 ± 8	92 ± 13
Liver (% body weight)	2.7 ± 0.6	2.5 ± 0.2	2.7 ± 0.4	2.5 ± 0.6	2.4 ± 0.5
Liver lipids (mg/g)					
total chol.	2.8 ± 0.3[b,c]	2.1 ± 0.1[b]	2.3 ± 0.3[c]	2.6 ± 0.3	2.6 ± 0.2
free chol. (FC)	1.5 ± 0.1[b,g]	1.6 ± 0.1[d,f]	1.5 ± 0.1[c,e]	1.9 ± 0.1[e,g,f]	2.1 ± 0.2[b,c,d]
esterified chol. (EC)[i]	2.1 ± 0.3[b,c,d]	0.8 ± 0.1[c]	1.3 ± 0.3	1.2 ± 0.3[d]	0.8 ± 0.3[b]
% FC	54.8 ± 3.5[b,c,d,e]	77.6 ± 2.9[f]	67.5 ± 5.3[d,f,g]	72.2 ± 4.9[e,h]	82.1 ± 3.8[d]
FC/EC	1.3 ± 0.2[b]	1.3 ± 0.2[c]	2.5 ± 0.5[c]	3.2 ± 0.8	5.9 ± 1.2[b,c]
phospholipid	13.8 ± 0.7[b,d,f]	19.1 ± 1.3[d]	14.8 ± 1.7[c,e]	20.7 ± 1.9[e,f]	21.2 ± 1.6[b,c]

[a]Values bearing the same letter in a given row are significantly different ($p < 0.05$) by the student t-test. For dietary group abbreviations, see text.

[i]Esterified cholesterol determined as free cholesterol and corrected by the factor 1.67.

DISCUSSION

Previous studies have implicated the atherogenic nature of animal protein as well as certain refined carbohydrates when fed either to rabbits, primates or humans (17-20). In most of these studies the total fat content of the diets used was in excess of 14%, with appreciable amounts being of the saturated type. While one study (17) used a diet low in fat (2.33%) provided by corn oil, in this experiment casein and dextrose were the only sources of protein and carbohydrate used. The results reported in the present work represent an initial attempt to

TABLE 5

Per cent Distribution of Serum Cholesterol Among Lipoprotein Classes in Rabbits. Values in parentheses represent mg cholesterol/mg (mean ± S.E.)[a]

Density (g/ml)	Dietary Group				
	CD	SD	CS	SS	C
VLDL (d < 1.006)	10.2 ± 2.6	15.3 ± 2.9	6.1 ± 1.7	16.6 ± 1.9	10.4 ± 3.9
	(18.0 ± 2.4)	(7.2 ± 2.1)	(15.8 ± 3.5)	(7.1 ± 1.7)	(8.5 ± 0.8)
IDL (d = 1.006-1.019)	6.7 ± 1.5	15.2 ± 1.0[c]	15.3 ± 2.5[c]	13.4 ± 0.9[c]	5.8 ± 1.0
	(12.1 ± 1.5)	(7.5 ± 1.0)	(39.6 ± 13.7)	(6.0 ± 0.7)	(5.0 ± 0.8)
LDL (d = 1.019-1.063)	59.5 ± 4.3[c]	30.4 ± 2.1	58.3 ± 4.9[c]	34.9 ± 4.4	29.2 ± 5.6
	(107.2 ± 25.6)	(14.8 ± 3.8)	(152.0 ± 37.6)	(14.2 ± 1.7)	(23.2 ± 4.0)
HDL (d = 1.063-1.21)	19.5 ± 2.0[c]	34.7 ± 2.0[b]	18.5 ± 3.6[c]	30.6 ± 3.5[b]	45.5 ± 5.2
	(35.4 ± 3.2)	(16.9 ± 2.3)	(47.9 ± 7.2)	(13.2 ± 1.1)	(37.3 ± 2.5)
R_f (d < 1.21)	4.6 ± 0.4	4.3 ± 0.8	1.9 ± 0.4	4.4 ± 0.6	9.1 ± 1.4
	(8.0 ± 0.9)	(2.2 ± 0.2)	(4.6 ± 0.5)	(1.9 ± 0.5)	(7.5 ± 3.1)
HDL/LDL	0.32	1.10	0.31	0.91	1.67
Total serum chol. (mg/dl)	181 ± 30	48 ± 9	259 ± 73	43 ± 4	82 ± 5

[a]Results are expressed as per cent of total serum cholesterol in each lipoprotein class and the residual fraction (R_f). Recovery of cholesterol averaged 93 ± 3%.

[b]Significantly different from chow-fed rabbits ($p < 0.01$).

[c]Significantly different from chow-fed rabbits ($p < 0.001$).

document the contributions of various protein and carbohydrate sources in the development of hypercholesterolemia and other lipid modifications in serum and liver tissue when fed to rabbits as part of a low fat, low cholesterol semi-purified diet. The changes in lipid concentrations reported here may be important in the development of atherogenesis in rabbits fed similar diets for longer periods (17).

With regard to the casein containing diets, we have verified the hypercholesterolemic nature of this animal protein in the rabbit. When casein diets were used, the higher total serum cholesterol concentrations were due to increases in both the free and esterified forms and an increase in the relative amounts of serum free cholesterol. In the livers of these animals, increases in the esterified cholesterol content only were observed causing a decrease in the percentage free cholesterol. These findings raise the possibility that hepatic receptors required for the uptake of cholesteryl ester containing lipoproteins may be saturated or suppressed in the casein fed case permitting the accumulation of total cholesterol, both free and esterified, in the circulation (21,22). This receptor phenomenon may account for the increased distribution of cholesterol in the serum LDL fraction and lower amounts in the HDL fraction when casein is fed. Further, within the casein fed groups the sucrose containing diet (CS) appears to add to this net effect, resulting in the increased distribution of both IDL and LDL cholesterol in the serum of these animals when compared with the CD diet fed rabbits. This finding may be due, in part, to the known lipogenic nature of sucrose (23) whereby increased amounts of triglycerides would be incorporated into the serum VLDL (1.006 g/ml) fraction. In previous studies, casein fed rabbits have been shown to secrete increased amounts of VLDL (24) which is cholesterol-rich (17). Consequently, the combination of casein and sucrose in the diet might be such that increased amounts of cholesterol-rich VLDL are produced. These VLDL may subsequently be transformed into cholesterol-rich IDL via the action of lipoprotein lipase (25). Also consistent with this mechanism is the observation that casein fed rabbit livers contain lower concentrations of phospolipids, which may be the result of increased hepatic demand for this polar lipid in VLDL synthetic processes. Further studies on this aspect would be most interesting.

Results from the feeding of the soy protein isolate-based diets verified the hypocholesterolemic effect of this protein in the rabbit as well (18). Reasons for this effect have been reviewed by Sugano (26). Briefly, in soy protein fed rabbits, cholesterol dynamics appear to be shifted in the direction of a lowered cholesterol pool size in both the plasma and tissues. The contribution of sucrose in producing elevations in serum cholesterol when fed as part of the soy protein based diet (SS) appears to be minimal. Some small differences between the SD and SS diets were apparent, however, The elevation of serum triglyceride concentrations on days 17 and 52 in the case of the SS diet fed rabbits once again points to the lipogenic nature of this sugar. When the SD diet was fed, it is unclear why lower liver cholesteryl ester concentrations

were observed, however.

No significant differences were seen in the distribution of cholesterol due to dietary carbohydrate type in the soy protein fed animals. Generally soy protein feeding caused significantly more cholesterol to be found in the IDL fraction but not in the LDL fraction as in the casein fed case. A possible reason for this finding is the presence of normal, functional endogenous lipoprotein receptors in the soy protein fed rabbit liver. Further studies are needed to clarify this possibility.

The calculation of HDL cholesterol/LDL cholesterol has been reported as a useful indicator of risk for atherosclerosis in man (27). In the present study, this ratio was markedly reduced in both casein fed rabbit groups when compared with their soy-protein counterparts or chow fed animals. It may therefore be a useful indicator in assessing the atherogenicity of the diets used.

When absolute levels of LDL and HDL cholesterol are considered, the findings are less distinct. In the case of CS diet fed rabbits, increases of both LDL and HDL cholesterol occurred; in the CD diet fed animals, only LDL cholesterol was elevated. Although a causal relationship between LDL levels and coronary heart disease has been well established, no animal or human studies have yet shown that raising the concentration of HDL will prevent coronary disease. The effect of hypercholesterolemia and increased LDL cholesterol concentrations might indeed override any beneficial effect of moderate HDL cholesterol elevations in the CS dietary case.

In the soy-protein fed group absolute amounts of HDL cholesterol were the lowest among rabbits fed any of the diets in this study. Low levels of HDL cholesterol, however, may not necessarily indicate higher atherogenic risk. Epidemiological studies among African and Asian populations have observed low levels of total plasma cholesterol, LDL cholesterol and HDL cholesterol along with a low incidence of coronary heart disease and atherosclerosis (28). It may be that the lower levels of total plasma cholesterol or LDL cholesterol are once again important determinants of risk in this regard. In either case, however, the use of HDL cholesterol/LDL cholesterol values still appears to be a useful index of risk for atherosclerosis.

Finally, it should be noted that discrepancies among other workers exist in the reported distributions of cholesterol among lipoprotein fractions in rabbits fed casein or soy protein containing diets. Terpstra et al. (29) have shown that in some rabbits an LDL band within the range of IDL as defined for humans (1.006-1.019 g/ml) is seen. Thus, the possibility has been raised that individual rabbits may differ in their response to the feeding of semi-purified diets. Roberts et al. (30) reported that the increase of serum cholesterol in casein fed rabbits was mostly due to increased levels in the IDL fraction.

Other workers (29,31,32) have observed higher levels of cholesterol in the LDL fractions compared with the IDL as reported in the present work. Ross et al. (17) found a similar distribution but with an increased amount of cholesterol in the VLDL fraction as well. Although the lipoprotein isolation technique used in the present study was essentially that used by Ross et al. (17), it should be noted that these workers used a diet containing 63.2% sucrose with no fiber. This additional amount of dietary sucrose may have accounted for the higher distribution of serum cholesterol in the VLDL by the production of greater amounts of cholesteryl-rich lipoproteins within this density class.

Further studies designed to assess the contribution of semi-purified diets in the development of hypercholesterolemia and atherogenesis in the rabbit should consider the individual responses of the rabbit to these types of diets. It is also felt, however, that experiments in which the effect of a specific non-protein dietary component is being evaluated for its ability to produce hypercholesterolemia in the rabbit should avoid casein as a sole protein source.

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REFERENCES

1. Carroll, K.K. (1978) Nutr. Rev. 36, 1-5.
2. Carroll, K.K. (1982) in New Trends in Nutrition, Lipid Research and Cardiovascular Diseases (Bazan, N.G., Paoletti, R., and Iacono, J.M., eds.) pp. 167-177, Alan R. Liss, Inc., New York, NY.
3. Sirtori, C.R., Gatti, E., Mantero, O., Conti, F., Agradi, E., Tremoli, E., Sirtori, M., Fraterrigo, L., Tavassi, L., and Kritchevsky, D., (1979) Am. J. Clin. Nutr. 32, 1645-1658.
4. Scholz, K.E., Beyman, A.C., and West, C.E. (1983) Ernahrungs Wiss 22, 85-96.
5. Huff, M.W., and Carroll, K.K. (1982) J. Nutr. 110, 1676-1685.
6. Carroll, K.K., and Hamilton, R.M.G. (1975) J. Food Sci. 40, 18-23.
7. Kritchevsky, D., Tepper, S.A., and Kitagawa, M. (1973) Nutr. Reports Int. 7, 357-362.
8. Kritchevsky, D., Sallata, D., and Tepper, S.A. (1968) J. Atero. Res. 8, 697-703.

9. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497-509.
10. Albers, R.W., and Lowry, D.H. (1955) Anal. Chem. 27, 1829-1831.
11. Bartlett, G.R. (1959) J. Biol. Chem. 234, 466-468.
12. Bauer, J.E., and Ransone, W.D. (1983) Lipids 18, 397-401.
13. Hatch, F.T., and Lees, R.S. (1968) in Advances in Lipid Research (Paoletti, R., and Kritchevsky, D., eds) Vol. 6, pp. 33-36, Academic Press, New York, NY.
14. Huff, M.W., Roberts, D.C.K., and Carroll, K.K. (1982) Athero. 41, 327-336.
15. West, C.E., Deuring, K., Schutte, J.B., and Terpstra, A.H.M. (1982) J. Nutr. 112, 1287-1295.
16. Anonymous (1977) Nutritional Requirement of Rabbits, 2nd ed, National Research Council, National Academy of Sciences, Washington.
17. Ross, A.C., Minick, C.R., and Zilversmit, D.B. (1978) Athero. 29, 301-315.
18. Carroll, K.K. (1982) Federation Proc. 41, 2792-2796.
19. Story, J.A. (1982) Federation Proc. 41, 2797-2800.
20. Van Raaij, J.M.A., Katan, M.B., and West, C.E. (1983) in Animal and Vegetable Proteins in Lipid Metabolism and Atherosclerosis (Gibney, M.J., and Kritchevsky, D., eds.) pp. 111-134, Alan R. Liss, Inc., New York, NY.
21. Kovanen, P.T., Brown, M.S., Basu, S.K., Bilheimer, D.W., and Goldstein, J.L. (1981) Proc. Nat. Acad. Sci. 78, 1396-1400.
22. Goldstein, J.L., Kita, T., and Brown, M.S. (1983) New Eng. J. Med. 309, 288-296.
23. Macdonald, I. (1966) in Advances in Lipid Research (Paoletti, R., and Kritchevsky, P., eds.) Vol. 4, p. 57, Academic Press, New York, NY.
24. Kritchevsky, D., Tepper, S.A., Czarnecki, S.K., Klurfeld, D.M., and Story, J.A. (1983) in Animal and Vegetable Proteins in Lipid Metabolism and Atherosclerosis (Gibney, M.J., and Kritchevsky, D., eds.) pp. 85-100, Alan R. Liss, New York, NY.
25. Nilsson-Ehle, P., Garfinkel, A.S., and Schotz, M.C. (1980) Ann. Rev. Biochem. 49, 667-693.
26. Sugano, M. (1983) in Animal and Vegetable Proteins in Lipid Metabolism and Atherosclerosis (Gibney, M.J., and Kritchevsky, D., eds.) pp. 51-84, Alan R. Liss, New York, NY.
27. Narayan, K.A. (1981) in High Density Lipoproteins (Day, C.E., ed.) pp. 588-591, Marcel Dekker, Inc., New York, NY.
28. Gotto, A.M., and Rifkind, B.M. (1982) in Atherosclerosis Reviews (Gotto, A.M., and Paoletti, R., eds.) Vol. 9, p. 7, Raven Press, New York, NY.
29. Terpstra, A.H.M., and Sanchez-Muniz, F.J. (1981) Athero. 39, 217-227.
30. Roberts, D.C.K., Huff, M.W., and Carroll, K.K. (1979) Lipids 14, 566-571.
31. Beynan, A.C., den Engelsman, G., Scholz, K.E., and West, C.E. (1983) Ann. Nutr. Metab. 27, 117-124.
32. Lacombe, C., and Nibbelink, M. (1981) Artery 6, 280-289.

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Inhibition of Fatty Acid Synthesis in Isolated Adipocytes by 5-(Tetradecyloxy)-2-Furoic Acid[1]

DAVID L. HALVORSON and **SYLVIA A. McCUNE***, Department of Biological Chemistry and Structure, University of Health Sciences/The Chicago Medical School, North Chicago, IL 60064

ABSTRACT

The compound 5-(tetradecyloxy)-2-furoic acid (TOFA), a hypolipidemic agent, inhibits fatty acid synthesis, lactate and pyruvate accumulation and CO_2 release in isolated rat adipocytes. TOFA stimulates the accumulation of citrate. ATP levels are not lowered by TOFA. In comparison with the natural fatty acid, oleate, TOFA exhibited a much greater inhibitory effect on lipogenesis. TOFyl-CoA formation within intact adipocytes was demonstrated. Although not inhibited by TOFA, acetyl-CoA carboxylase is inhibited by TOFyl-CoA. It is proposed that many of the metabolic effects of TOFA in isolated adipocytes can be explained by TOFyl-CoA inhibition of acetyl-CoA carboxylase. TOFA inhibits glycolysis as a secondary event with the primary event of inhibition of fatty acid synthesis causing an accumulation of citrate which is an inhibitor of phosphofructokinase.
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INTRODUCTION

Fatty acid synthesis in both liver and adipose tissue is known to be inhibited by various long chain fatty acids (1,2). The mechanism of this inhibition is through the action of the CoA esters of the fatty acids on acetyl-CoA carboxylase (3,4), which is generally considered the rate limiting enzyme in fatty acid synthesis. The degree of inhibition varies depending on the particular fatty acid (2). It has been demonstrated that 5-(tetradecyloxy)-2-furoic acid (TOFA or MDL 14,514) inhibits lipogenesis both in vivo and in isolated hepatocytes (5-8). This compound is structurally very similar to long chain fatty acids (9). The inhibition of fatty acid synthesis was shown to be through TOFyl-CoA, the CoA derivative of TOFA (8,9), and it is TOFyl-CoA, not TOFA, that has been demonstrated to inhibit acetyl-CoA carboxylase to a greater extent than oleyl CoA (9).

There is an observed decrease in glycolytic activity in hepatocytes treated with TOFA (8). The concentrations of glucose-6-phosphate and fructose-6-phosphate are increased while the levels of pyruvate, fructose-1,6-diphosphate and lactate are shown to decrease. This indicates an inhibitory action on the enzyme phosphofructokinase. A possible explanation for this observation is that the increased citrate concentration seen in TOFA treated cells causes the inhibition of phosphofructokinase.

It is well-established that a number of long-chain fatty acids inhibit adipose tissue acetyl-CoA carboxylase (10,11). Therefore, because of the similarity of TOFA to a long-chain fatty acid, we examined the metabolic effects of TOFA on adipocytes isolated from rat epididymal fat pads. The mechanism of action of TOFA in adipocytes will be compared with that observed in hepatocytes. A preliminary report of the work has been presented previously (12).

*To whom correspondence should be addressed.

MATERIALS AND METHODS

Adipocytes were isolated between 10:00 and 11:00 a.m. from the epididymal fat pads of "ad libitum" fed male Sprague-Dawley rats (150-220 g) by the method of Rodbell (13). Incubations were carried out in 20 ml plastic incubation vials in a shaking water bath at 37 C under 95% O_2/5% CO_2 atmosphere. The cells were suspended in a final volume of 2.0 ml in a reduced Ca^{2+} (1.3 mM) Krebs-Henseleit buffer (pH 7.4) with 2.0% bovine serum albumin (Fraction V, Sigma Chemical Co., charcoal treated and dialyzed), 3.0 mM glucose, 0.04 ml insulin (0.1 mg/ml) and 0.05 ml bacitracin (1.0 mg/ml). Aliquots of TOFA dissolved in acetone were added to the incubation vials and allowed to dry before preincubation with the buffer/bovine serum albumin mixture, a procedure described by Panek et al. (7) which causes all of the TOFA to be dissolved.

Fatty acid synthesis was measured by the incorporation of ^3H_2O (0.5 mCi/ml) into fatty acids. The cells were terminated by the addition of 0.1 ml 60% $HClO_4$ and extracted twice for total lipids with 2:1 methanol-chloroform at 5 C for 24 hr as described by Kates (14). Further extraction of the samples was according to Harris (15). The fatty acid synthesis rate was determined as described by Jungas (16).

$^{14}CO_2$ release was determined by the method

of Crabb and Harris (17) using [U-^{14}C]glucose (0.25μCi/ml) in the incubation. Metabolites were measured in KOH-neutralized $HClO_4$ extracts of cell preparations. The assays were conducted spectrophotometrically by enzymatic methods according to Hohorst et al. (18) for lactate and pyruvate, Mollering and Gruber (19) for citrate and Lamprecht and Trautschold (20) for ATP.

For the measurement of the formation of TOFyl-CoA, the isolated adipocytes were incubated for 90 min with or without TOFA and then 2 different methods were used to confirm the presence of TOFyl-CoA. With one procedure at the end of the incubation period the cells were washed 3 times with fresh buffer terminated with 2:1 methanol-chloroform, and extracted for 24 hr at 5 C. TOFyl-CoA was assayed spectrophotometrically as described by Harris and McCune (9). The second method of identification of TOFyl-CoA was by the basic procedure of McGee and Spector (2). The sample obtained was chromatographed on Silica Gel G thin layer plates in petroleum ether/ether/acetic Acid (70/30/1, v/v/v). After air drying, the silica gel in a band 0.5 cm on either side of the origin was scraped into screw cap test tubes and then incubated with 5 ml 0.3N NaOH in 90% methanol for 30 min at 73 C to hydrolyze any TOFyl-CoA. The samples were cooled, 0.5 ml 5 N HCl added, and then extracted 3 times with 5 ml aliquots of petroleum ether. The combined extracts were taken to dryness under N_2 and then taken up in a small volume of acetone and chromatographed as described above with TOFA, oleate and palmitate standards. After air drying, the plate was sprayed with dilute H_2SO_4 and heated to detect the fatty acids. Under these conditions oleate or palmitate exhibit an R_F of 0.69 while TOFA has an R_F of 0.45 and all CoA esters of these compounds remain at the origin.

Radioactive compounds were obtained from Research Products Int. Type II collagenase, other enzymes, and most biochemicals were obtained from Sigma Chemical Co. TOFA (MDL 14,514) was a gift from Merrell Dow Pharmaceuticals, Inc. (Cincinnati, Ohio).

RESULTS

Effect of TOFA on the Fatty Acid Synthesis Rate

TOFA inhibits fatty acid synthesis in isolated adipocytes. This inhibition was observed in cell suspensions incubated with TOFA concentrations ranging from 5-250 μM (Fig. 1). This concentration-dependent effect was nearly maximal at 50 μM. Further inhibition of fatty acid synthesis by higher concentrations of TOFA seemed to be minimal.

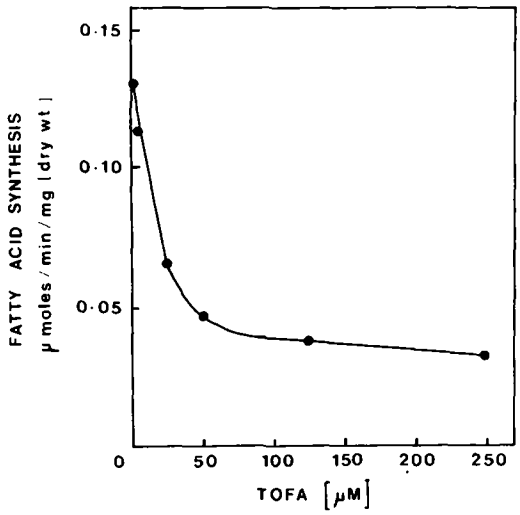

FIG. 1. Inhibition of fatty acid synthesis of adipocytes prepared from male rat epididymal fat pads as a function of TOFA concentration. Incubations were conducted with 50 mg. wet wt. of adipocytes/ml. Insulin and bacitracin were present in all incubations. Tritiated water was added after 30 min of incubation. Incorporation of ^3H_2O into fatty acids was determined after an additional 60 min of incubation. A representative experiment is presented which was reproduced with another preparation of adipocytes.

The presence of insulin had no significant effect on the inhibition of lipogensis observed in cells incubated with TOFA (Table 1). The inhibitory effect was substantial in both cases. TOFA had a much greater inhibitory capacity than the naturally occurring fatty acid oleate. Adipocytes incubated with 50 μM TOFA showed a 62% decrease of fatty acid synthesis, while cells incubated with 100 μM oleate exhibited no significant decrease in lipogenic rate (Table 1). Cells incubated with 500 μM TOFA showed an 81% inhibition, while 500 μM oleate reduced lipogenesis by only 23%. These levels of inhibition by TOFA are in agreement with studies using hepatocytes (8).

Effects of TOFA on Cellular Processes and Metabolite Concentrations

The effects TOFA had on cellular processes and metabolite concentrations present in the adipocyte are shown in Table 2. Cells incubated with 50 μM TOFA were observed to show a significant decrease in the amount of $^{14}CO_2$ released and fatty acid synthesis. The concentration of pyruvate present in cells incubated with TOFA also was shown to decrease significantly

TABLE 1

The Comparative Inhibition of Lipogenesis in Isolated Adipocytes by TOFA and Oleate

Additions	Rate of Fatty Acid Synthesis (μMoles/min/mg dry wt.)	
	Basal rate (no insulin)	+ Insulin
None	0.029 ± 0.007	0.232 ± 0.027
TOFA (50 μM)	0.005 ± 0.002*	0.066 ± 0.008*
TOFA (500 μM)	0.002 ± 0.001*	0.022 ± 0.009*
Oleate (100 μM)	–	0.225 ± 0.030
Oleate (500 μM)	–	0.186 ± 0.024*

Note: Adipocytes (60-100 mg dry wt/ml) were pre-incubated 30 min with each agent at 37 C prior to introduction of the ^3H_2O radiolabel. Incubation period with ^3H_2O lasted 60 min. Shown are mean ± SEM for 3 experiments.

*Values significantly different by Student's t test for paired samples from the same conditions without drug, $P<0.05$.

TABLE 2

The Effect of TOFA on Metabolite Concentrations and CO_2 Release in Isolated Adipocytes

Process or metabolite	Control	TOFA 50 μM
$^{14}CO_2$ Release[a]	6463 ± 257	2824 ± 702*
Pyruvate[b]	1.02 ± 0.10	0.81 ± 0.06*
Lactate[b]	5.52 ± 2.78	4.58 ± 1.86
ATP[b]	0.24 ± 0.06	0.21 ± 0.03
Citrate[b]	0.22 ± 0.03	0.28 ± 0.02*

Note: Adipocytes (75-100 mg dry wt/ml) were preincubated for 30 min in the presence of insulin and bacitracin at 37 C. After another 60 min of incubation, the samples for metabolites and CO_2 release were taken as described in Materials and Methods. Results are expressed as means ± SEM for 3 preparations of adipocytes.

[a]Release expressed as counts per minute.

[b]All metabolites measured as nmoles per mg dry wt of cells.

*Values significantly different by student's t test for paired samples from the same conditions without drug, $p<0.05$.

while the citrate concentration was shown to increase significantly. These observations correlate with those made in TOFA-treated hepatocytes (8). Lactate concentrations were lowered, but not significantly. ATP concentration was not altered in adipocytes incubated in the presence of TOFA. As the TOFA concentration present during the incubation increased, the metabolic effects on the cells were more dramatic (Fig. 2).

In a time study of these effects (Fig. 3A, B,C), cells incubated with TOFA showed a rapid effect (as early as 15 min) on lowering the rate of fatty acid synthesis in cell suspensions terminated at various times. However, the pyruvate concentration was observed to increase initially, stabilize, and then decrease while, simultaneously, the citrate concentration remained stable and increased after 45 min of incubation. A similar time course and change in metabolic concentrations occur in hepatocytes (McCune–unpublished observations).

Formation of TOFyl-CoA

The proposed action on lipogenesis is through the inhibition of acetyl-CoA carboxylase by the CoA derivative of TOFA. Two lines of evidence

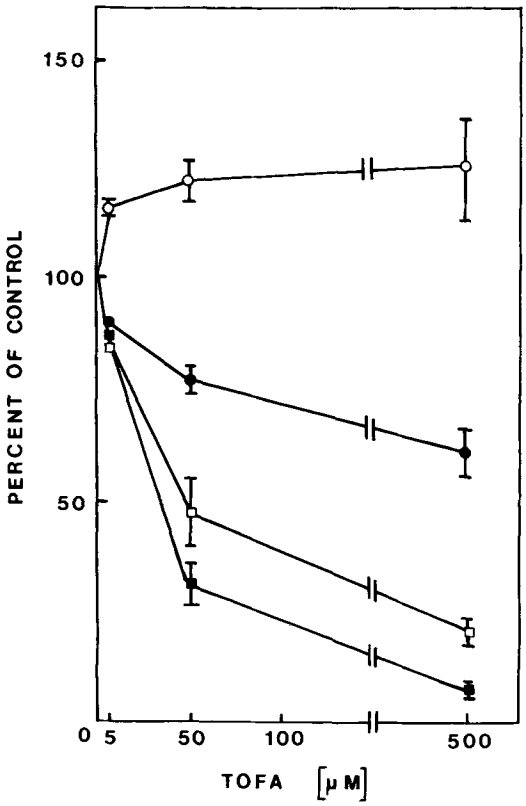

FIG. 2. Effects of increased concentrations of TOFA on fatty acid synthesis and metabolite concentrations. Adipocytes (80-100 mg dry wt/ml) were pre-incubated for 30 min. Insulin and bacitracin were present in all incubations. Control cells were not incubated in the presence of TOFA, but experimental cells were incubated in the presence of various levels of TOFA. ^3H_2O was added after the pre-incubation period of 30 min, and rates of fatty acid synthesis (■) were calculated by ^3H_2O incorporation into fatty acids after an additional 60 min of incubation. Citrate (○), pyruvate (●) and $^{14}CO_2$ (□) release were determined as described in Materials and Methods. Results are shown as mean ± SEM for 3 experiments.

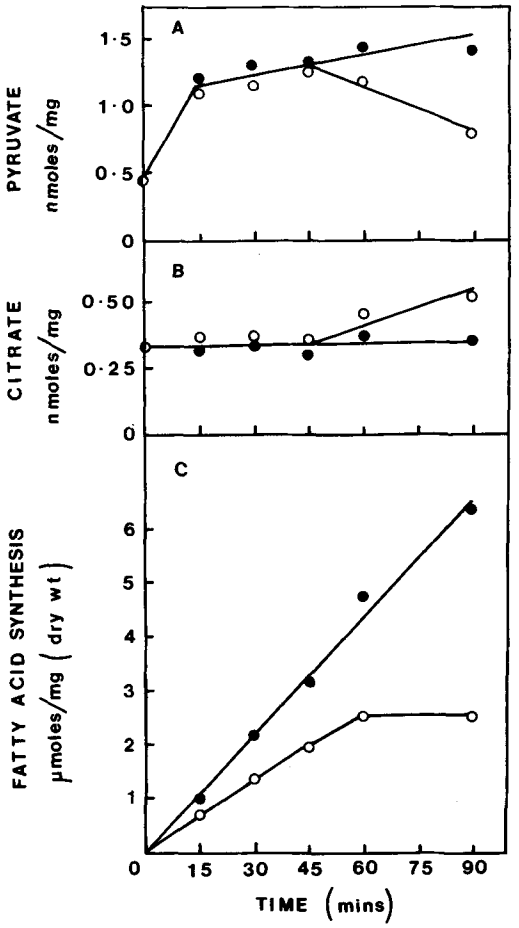

FIG. 3. Changes (with respect to time) in fatty acid synthesis, citrate and pyruvate levels in adipocytes treated with 50 μM TOFA. Adipocytes (85 mg dry wt/ml) were incubated in the presence of insulin and bacitracin for up to 90 min. ^3H_2O was added at zero time for determination of fatty acid synthesis. Control incubations–(●); TOFA-treated cells–(○). Results shown here are data from a representative experiment which was reproduced with 2 other preparations of adipocytes.

indicate that TOFyl-CoA is formed in the adipocyte. The CoA ester of TOFA has a distinctive spectrum (9) containing 2 peaks, one at 259 nm (ε mM = 14.3), which corresponds to the adenine moiety of the CoA portion of the molecule, and the other at 326 nm (ε mM = 17.2), which corresponds to the shift caused in the absorbance maximum of TOFA as a result of formation of the thiol ester bond. The 2:1 methanol-chloroform extracts of cells incubated with TOFA were measured spectrophotometrically against extracts of non-TOFA treated cells. The spectrum observed contained 2 absorbance peaks. The first peak was observed at 259 nm, while the second peak had maximum absorbance at 326 nm, which indicated the formation of TOFyl-CoA in adipocytes incubated with TOFA (data not shown). Using the molar extinction coefficient for TOFyl-CoA, the concentration of TOFyl-CoA formed by the cells was 1.03 nM per mg dry weight of adipocytes present during the incubation. Thin layer chromatography (TLC) also demonstrated that extracts isolated from cells incubated with TOFA form a CoA ester of TOFA, because when the material taken from the origin of the first thin layer plate is hydrolyzed with sodium hydroxide, TOFA appears on the second plate

only in samples obtained from cells which were incubated with TOFA (zero time controls with TOFA do not exhibit a TOFA band with the second thin layer).

DISCUSSION

In this study, we show that TOFA inhibits fatty acid synthesis in isolated adipocytes in a concentration-dependent manner, with near maximal inhibition occurring at 50 μM. This inhibition is not significantly altered by the presence of insulin. TOFA has been shown to be a more potent inhibitor of fatty acid synthesis than the naturally occurring fatty acid, oleate. Reasons for this difference may be that TOFyl-CoA has greater binding to acetyl-CoA carboxylase than oleyl-CoA, or TOFA is more readily esterified to the CoA form or less easily oxidized than is oleate. A further explanation may be that TOFA and/or TOFyl-CoA may not be sequestered as readily by the fatty acid binding protein (21).

Other significant metabolic changes were observed in adipocytes incubated with TOFA. There was a small but significant rise in the concentration of citrate. This would be expected if acetyl-CoA carboxylase was inhibited by TOFyl-CoA. The citrate increase may cause a decrease in the glycolytic rate through an inhibition of phosphofructokinase (22). Criticism of this idea may be that the increase in the citrate concentration is not large enough to inhibit glycolysis at phosphofructokinase. A study by Saggerson and Greenbaum (23) showed that adipocytes incubated with insulin and epinephrine showed a 480% increase in cellular citrate concentration but no inhibition of phosphofructokinase. However, addition of oleate to the mixture did cause some inhibition, and they concluded that the citrate was compartmentalized within the cell, probably in the mitocondrion. McCune and Harris (8) observed an increased flux of citrate from the mitochondria of isolated hepatocytes incubated with TOFA which probably also occurs in adipocytes. Therefore, TOFA added to adipocytes not only inhibits acetyl-CoA carboxylase which causes increased citrate levels, but probably inhibits phosphofructokinase by causing a large enough efflux of citrate from the mitocondria to attain the threshold necessary for the inhibition of this enzyme.

The time study concerning the effect of TOFA on the glycolytic activity gave further evidence concerning a crossover at the point of phosphofructokinase. It seems that there is an immediate effect by TOFA on fatty acid synthesis but the effects observed on the pyruvate and citrate concentration show a definite lag period between the early and later incubation times. It may be that citrate is required to reach a certain concentration before the effects on the glycolytic pathway can be observed. Further support for the inhibition of glycolysis is the inhibition of $^{14}CO_2$ release, which was observed in cells incubated with TOFA. An inhibition of $^{14}CO_2$ release of 56% was observed in adipocytes incubated with 50 μM TOFA.

It has been shown that the CoA ester of TOFA will inhibit acetyl-CoA carboxylase purified from isolated hepatocytes (9). TOFA itself has no inhibitory effect on the enzyme. The presence of TOFyl-CoA in rat adipocytes incubated with TOFA, indicated by our spectrophotometric and chromatographic findings, suggests that the inhibition of fatty acid synthesis by TOFA is mediated through a CoA derivative in these cells. Thus, the inhibition of lipogenesis in adipocytes has characteristics of the inhibition that has been observed in hepatocytes (8,9).

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REFERENCES

1. Numa, S., Bortz, W.M., and Lynen, F. (1965) Advan. Enzyme. Regul. 3, 407-423.
2. McGee, R., and Spector, A.A. (1975) J. Biol. Chem. 250, 5419-5425.
3. Bortz, W.M., and Lynen, F. (1963) Biochem. Z 337, 505-509.
4. Ogiwara, H., Tanabe, T., Nikawa, J., and Numa, S. (1978) Eru. J. Biochem. 89, 33-41.
5. Parker, R.A., Kariya, T., Grisar, J.M., and Petrow, V. (1977) J. Med. Chem. 20, 781.
6. Kariya, T., and Willie, L.J. (1978) Biochem. Biophys. Res. Commun. 80, 1022-1024.
7. Panek, E., Cook, G.A., and Cornell, N.W. (1977) Lipids 12, 814-818.
8. McCune, S.A., and Harris, R.A. (1979) J. Biol. Chem. 254, 10095-10101.
9. Harris, R.A., and McCune, S.A. (1981) Methods of Enzymology 72, 557-559.
10. Denton, R.M. (1975) Proc. Nutr. Soc. 34, 217-224.
11. Volpe, J.J., and Vagelos, P.R. (1976) Physiol. Rev. 56, 339-417.
12. Halvorson, D., and McCune, S.A. (1983) Fed. Proc. 42, 1854.
13. Rodbell, M. (1964) J. Biol. Chem. 239, 375-384.
14. Kates, M. (1972) Techniques of Lipidology, Isolation, Analysis, and Identification of Lipids, (Kates, M., ed.), North Holland, Amsterdam, p. 349 and 363.
15. Harris, R.A. (1975) Arch. Biochem. Biophys. 169, 168-180.
16. Jungas, R.G. (1968) Biochemistry 7, 3708-3717.
17. Crabb, D.W., and Harris, R.A. (1978) J. Biol. Chem. 253, 1481-1487.

18. Hohorst, J.J., Krentz, F.H., and Bucher, T. (1959) Biochem. Z., 332, 18-46.
19. Mollering, H., and Gruber, W. (1966) Anal. Biochem. 17, 369-379.
20. Lamprecht, W., and Trautschold, I. (1974) Methods of Enzymatic Analysis, (Bergmeyer, H.V., ed.), 2nd Ed., Academic Press, New York, pp. 2101-2109.
21. Lunzer, M.A., Manning, J.A., and Ockner, R.K. (1977) J. Biol. Chem. 252, 5483-5487.
22. Bloxham, D.P., and Lardy, H.A. (1983) in The Enzymes, Vol. 8, (Boyer, P.D., ed.), Academic Press, New York, pp. 239-278.
23. Saggerson, E.D., and Greenbaum, A.L. (1970) Biochem. J. 119, 193-219.

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Lung Surfactant Phospholipids in Different Animal Species

SUE A. SHELLEY,* JUNE E. PACIGA and **JOHN U. BALIS,** *Department of Pathology, University of South Florida Medical Center, and James A. Haley Veterans Administration Hospital, Tampa, FL 33612*

ABSTRACT

A comparative study of adult mammalian lung surfactants was undertaken to determine which animal species might serve as appropriate models for surfactant alterations in human lung diseases. Phosphatidylcholine (PC) comprised 80-87% of the phospholipid and contained more than 65% palmitic acid in all species studied. Phosphatidylglycerol (PG) was found to vary significantly in fatty acid composition among the species. Rabbit, dog and rat surfactant PG contained 50-60% palmitic acid, while human and cat surfactant contained much lower levels of saturated fatty acids. Both the PC and PG of all species contained 2 positional isomers of fatty acids with 16 carbons and one double bond, but the relative amounts of the unusual isomer, 16:1Δ7, and palmitoleic acid, 16:1Δ9, varied among the different animal species. Only cat and dog surfactant phospholipids contained 18:1Δ5. Cat surfactant phospholipids also differed by the absence of 20:4 and the presence of small amounts of several 20- and 22-carbon fatty acids. These results explain some discrepancies found in the literature concerning surfactant composition and delineate limiting factors in extrapolating results from animal studies for the evaluation of maturation and pathological alterations in human surfactant.
Lipids 19:857-862, 1984.

INTRODUCTION

Lung surfactant has been investigated extensively since its discovery was reported by Pattle (1), its characteristic surface properties demonstrated by Clements (2), and its deficiency in infants with hyaline membrane disease (HMD) described by Avery and Mead (3). Animal models have been used to study the pathophysiology and treatment of surfactant deficiency on the assumption that this material is essentially identical in various species. Many reports of phospholipids in lung tissue, lung washings and type II cells from various species can be found in the literature and have been summarized in a recent review article (4). The phospholipid composition of purified surfactant fractions also has been reported for a number of species (5). However, in most cases only a single species has been studied carefully by each group of workers, using quite different methods for isolating and analyzing the surfactant. During the course of our study with human surfactant (6) we have conducted a systematic, comparative analysis of mammalian lung surfactants to determine whether characteristic compositional differences exist, thereby defining limiting factors of animal models used to investigate human lung disease. Our results clearly show differences in the phospholipid composition, as well as the fatty acid compositions of both phosphatidylcholine (PC) and phosphatidylglycerol (PG), among various animal species. A portion of this work has been published in abstract form (7).

*To whom correspondence should be addressed.

METHODS

Human lung tissue was obtained from pneumonectomy or lobectomy specimens as described previously (6). Lungs were taken from 6-7 individual dogs, cats and rabbits, and 7 groups of 10 rats. In addition, selected studies were performed using one group of 6 hamster lungs, and 2 bovine and porcine lungs. Using identical techniques, surfactant was isolated from minced lung tissues (8) by repetitive centrifugation on discontinuous sodium bromide density gradients as previously described (9). The visible, white particulate band of surfactant, located at a density of about 1.085 at 4 C, was removed and used for biochemical analyses of the phospholipids.

Lipids were extracted from the purified surfactants with chloroform/methanol (2:1, v/v) (10), and the total phospholipid content was determined by measuring the phosphorus content after digestion of the lipids with perchloric acid (11). The amount of phospholipid present was calculated on the assumption that the phospholipids contain an average of 4% phosphorus. Phospholipid classes were separated by two dimensional thin layer chromatography (TLC) on silica gel G plates using a basic solvent in the first dimension, chloroform/methanol/58% ammonium hydroxide (90:30:6, v/v/v) and an acidic solvent, chloroform/methanol/acetone/acetic acid/water (60:15:20:10:4, v/v/v/v/v) in the second dimension. The amount of each phospholipid present was determined using the method of Vaskovsky et al. (12) after spraying the TLC plates with 50% sulfuric acid

and charring. Disaturated PC (DSPC) was separated from the total lipid extract following reaction with osmium tetroxide using an alumina column and quantitated using an enzymatic method (13). No other phospholipids were detected in the DSPC extracts of purified surfactants evaluated in the TLC system described above. For fatty acid analysis, the TLC plates were sprayed with Rhodamine 6G, 0.01% in water, the PC and PG spots were detected under ultraviolet light, and then scraped from the plate. Fatty acid methyl esters were prepared by transmethylation (14) and quantitated by gas liquid chromatography (GLC) on a 10% EGSS-X column. The identity of unusual odd-numbered fatty acids was confirmed by GLC following catalytic hydrogenation. Further gas liquid chromatographic studies were carried out using a 50 meter Silar 10C glass capillary column.

The position of the double bond in an unusual 16:1 fatty acid was determined using dog lung surfactant. Hexadecenoic acid methyl esters were isolated by preparative GLC on a 10% EGSS-X column using a 10:1 post-detector splitter. Following ozonolysis and reductive cleavage with triphenylphosphine (15), the aldehydes and aldehyde esters were separated and identified by GLC on the Silar 10C capillary column.

RESULTS

As expected, PC was the most abundant phospholipid in all species and comprised from 80 to 87% of the total phospholipid (Table 1). Cat and rat surfactants contained significantly more PC ($p < 0.05$) than did human and dog surfactants. The average DSPC content of surfactants ranged from 53% of the total phospholipid in rat to 63% in cat. The PG content of cat surfactant varied from 1 to 5% of the total phospholipid, much less than that of all other species studied. This can be best appreciated from the PC/PG ratios shown in Table 1. Preliminary studies with surfactant purified from hamster, porcine and bovine lungs indicated that surfactant from these species did not differ from human surfactant in either PC or PG content.

Significant differences were found in the fatty acid compositions of both PC and PG (Tables 2 and 3). Both cat and dog surfactants contained less palmitic acid (16:0) and more oleic acid (18:1) in the PC than did human surfactant. In addition, cat surfactant PC contained less palmitoleic acid (16:1) than did human. Rabbit and rat surfactant PC did not differ from human in palmitic acid content, but differed in the contents of palmitoleic and the 18-carbon fatty acids. No 2 animal species were identical, and only rat surfactant contained as many saturated fatty acids in PC as did human surfactant.

PG fatty acid content varied even more greatly among the species than that of PC. The PG of human surfactant contained signifi-

TABLE 1

Surfactant Phospholipid Distribution—Per cent of Total Phospholipid (Mean ± SE, n = 6)

Phospholipids	Human	Cat	Dog	Rabbit	Rat
Phosphatidylcholine	80.5 (±1.4)	86.3[a] (±1.7)	81.3 (±1.4)	83.6 (±0.8)	87.0[a] (±0.8)
Phosphatidylglycerol	9.1 (±0.4)	2.1[a] (±0.7)	11.1 (±1.1)	8.0 (±0.5)	8.3 (±0.4)
Phosphatidylethanolamine	2.3 (±0.8)	3.2 (±0.8)	2.0 (±0.6)	3.5 (±0.4)	0.7 (±0.2)
All other phospholipids[b]	8.1 (±0.6)	8.4 (±1.0)	5.6[a] (±0.3)	4.9[a] (±0.6)	4.0[a] (±0.9)
$\frac{\text{Disaturated Phosphatidylcholine}}{\text{Total Phosphatidylcholine}} \times 100$	68 (±2)	73 (±5)	Not Done	69 (±5)	61 (±3)
$\frac{\text{Phosphatidylcholine}}{\text{Phosphatidylglycerol}}$	8.9 (±0.5)	52.5[a] (±12.2)	7.9 (±1.1)	10.7 (±0.7)	10.5 (±0.5)

[a]Significantly different from human surfactant with $p < 0.05$.

[b]Includes sphingomyelin, phosphatidylinositol (PI), phosphatidylserine (PS) and lysobis-phosphatidic acid. These phospholipids were determined separately in some samples, and no differences were observed among the species. The amounts of PI ranged from 1.5-2.8% and PS from 0.2-0.9%, while the amounts of the others were more variable.

TABLE 2

Fatty Acid Composition of Surfactant Phosphatidylcholine
Fatty Acid Distribution—Per cent of Total Fatty Acids (Mean ± SE)

	N =	Human 11	Cat 6	Dog 7	Rabbit 5	Rat 7
Major fatty acids (>1.5% of total fatty acids)	14:0	3.5 (±0.3)	3.8 (±0.7)	2.6 (±0.5)	2.3[a] (±0.2)	3.9 (±0.3)
	16:0	73.1 (±1.1)	65.7[a] (±0.7)	65.8[a] (±0.8)	71.6 (±1.8)	72.8 (±1.7)
	16:1	7.3 (±0.3)	2.8[a] (±0.2)	7.4 (±0.6)	5.5[a] (±0.7)	10.4[a] (±0.7)
	18:0	3.2 (±0.2)	3.9[a] (±0.2)	6.7[a] (±0.5)	1.6[a] (±0.1)	2.4[a] (±0.2)
	18:1	8.9 (±0.5)	13.9[a] (±0.6)	12.8[a] (±0.6)	11.9[a] (±0.6)	4.7[a] (±0.3)
	18:2	2.0 (±0.4)	3.0 (±0.3)	1.8 (±0.2)	5.7[a] (±1.0)	3.7[a] (±0.5)
Other fatty acids[b]		2.0	6.9	2.9	1.4	2.1
Per cent saturated fatty acids		81.3 (±1.0)	74.9[a] (±1.0)	76.7[a] (±0.9)	76.6[a] (±1.9)	80.0 (±1.8)
Per cent monounsaturated fatty acids		16.2 (±0.7)	18.2 (±0.7)	20.4[a] (±0.7)	17.7 (±1.0)	15.1 (±1.0)
Per cent polyunsaturated fatty acids		2.5 (±0.5)	6.9[a] (±0.5)	2.9 (±0.6)	5.7[a] (±1.0)	4.9[a] (±0.9)
Total saturated fatty acids / Total unsaturated fatty acids		4.6 (±0.3)	3.0[a] (±0.2)	3.3[a] (±0.2)	3.4[a] (±0.4)	4.2 (±0.5)

[a]Significantly different from human surfactant with $p < 0.05$.

[b]Includes from 0.1 to 1.2% of 15:0, 17:0 and 20:4. In addition, cat lung surfactant contained three 20-carbon and one 22-carbon unsaturated fatty acids in amounts ranging from 1.0 to 1.6% (see Fig. 1). These fatty acids were present in trace amounts or were not detectable in other species.

cantly less palmitic acid than all other species (Table 3), and only cat surfactant contained a similar low level of saturated fatty acids in the PG. However, cat surfactant differed from human surfactant in PG fatty acid composition in that it contained less palmitoleic, stearic (18:0) and oleic acids, and more palmitic, linoleic (18:2) and other unsaturated fatty acids.

Analysis of surfactant PC and PG fatty acids by capillary GLC revealed additional differences in unusual positional isomers of monounsaturated fatty acids (Fig. 1). Surfactant PC and PG in all species examined contained at least 2 positional isomers of fatty acids with 16 carbons and one double bond. One of these isomers is the commonly found palmitoleic acid, 16:1Δ9, which is the expected product of Δ9-desaturation of palmitic acid. The other isomer was identified by analysis of its ozonolysis products as 16:1Δ7. Human and rabbit surfactant PC contained 2-3 times more palmitoleic acid than 16:1Δ7, while in rat surfactant PC the two 16:1 isomers were approximately equal in amounts (Table 4). By contrast, cat and dog surfactant PC contained considerably more of the unusual 16:1 isomer than palmitoleic acid. The specificity of this fatty acid to the surfactant system was examined, using dog lung, by comparison of the 16:1Δ9/16:1Δ7 ratio in surfactant PC, total dog lung PC and dog liver PC. We found that while total lung PC contained less hexadecenoic acids than surfactant PC (4.4% vs. 7.3%) the ratio of 16:1Δ9/16:1Δ7 was the same. In liver PC only 1% of the fatty acids were hexadecenoic acids, and the two isomers were present in equal amounts.

Capillary GLC also allowed quantitation of 18:1Δ5 previously reported in dog surfactant (16). This fatty acid was found only in dog and cat surfactant and was not detected in any other species. In dog lung surfactant PC the

TABLE 3

Fatty Acid Composition of Surfactant Phosphatidylglycerol
Fatty Acid Distribution–Per cent of Total Fatty Acids (Mean ± SE)

	N =	Human 9	Cat 4	Dog 6	Rabbit 5	Rat 7
Major fatty acids (>1.5% of total fatty acids)	16:0	22.2 (±1.2)	36.0[a] (±4.7)	54.5[a] (±1.5)	50.5[a] (±4.0)	58.9[a] (±4.0)
	16:1	3.0 (±0.2)	0.8[a] (±0.1)	3.3 (±0.6)	3.2 (±0.6)	4.0[a] (±0.2)
	18:0	16.7 (±1.0)	6.3[a] (±1.2)	12.6[a] (±1.6)	5.3[a] (±0.2)	5.8[a] (±0.5)
	18:1	51.3 (±1.4)	31.0[a] (±2.4)	23.4[a] (±0.7)	33.5[a] (±1.5)	19.2[a] (±2.0)
	18:2	4.0 (±0.6)	11.2[a] (±2.1)	1.6[a] (±0.3)	5.0 (±0.7)	8.7[a] (±1.1)
Other fatty acids[b]		2.8	14.7	4.6	2.5	3.4
Per cent saturated fatty acids		40.0 (±1.7)	43.8 (±4.0)	69.2[a] (±0.8)	56.8[a] (±3.5)	65.7[a] (±3.7)
Per cent monounsaturated fatty acids		54.3 (±1.4)	35.6[a] (±2.5)	27.6[a] (±0.7)	37.5[a] (±3.6)	23.4[a] (±2.0)
Per cent polyunsaturated fatty acids		5.7 (±0.8)	20.6[a] (±2.5)	3.2 (±0.5)	5.7 (±0.4)	10.9[a] (±1.7)
Total saturated fatty acids / Total unsaturated fatty acids		0.7 (±0.05)	0.9 (±0.15)	2.3[a] (±0.10)	1.4[a] (±0.28)	2.2[a] (±0.35)

[a]Significantly different from human surfactant with $p < 0.05$.

[b]In all species except cat, other fatty acids includes from 0.1 to 1.4% 14:0, 15:0, 17:0, 20:4 and the same 22 carbon unsaturated fatty acid (22:x) found in cat phosphatidylcholine. All of these fatty acids are present in cat surfactant phosphatidylglycerol, with the 22:x being 2.8% of the total fatty acids. In addition, the 20-carbon unsaturated fatty acids present in cat surfactant PC also are present in the PG in amounts ranging from 1.4 to 3.8%.

ratio of 18:1Δ9 (oleic acid) to 18:1Δ5 was 0.9, while in surfactant PG it was 4.5. No 18:1Δ5 was detected in dog liver PC. In cat lung surfactant PC the ratio was 0.7 and in PG, 5.8.

DISCUSSION

Mammalian lung surfactants contain large quantities of dipalmitoylphosphatidylcholine (DPPC), the major component which allows generation of the low surface tension (<9 mN/m) found in the contracted lung (17). In the present comparative investigation we isolated surfactant from minced lung washings using techniques which were identical to those reported previously in a study of human surfactant (6). Therefore, the presented data provide a basis for a meaningful comparison with the human surfactant system. The surfactants from all species contained a large amount of PC, with palmitic acid comprising at least 65% of the total PC fatty acids. In a previous study of surfactants from human newborns with or without HMD we found that although PC values show a broad variation, 65% palmitic acid in the PC was the lowest value consistent with adequate neonatal lung function regardless of gestational age (18). These results and the PC fatty acid composition in different species suggest that, while a minimum level of DPPC is required for normal surfactant function, there is no apparent need for an exact metabolic control of each molecular species of PC.

Although the role of PG in surfactant function is not clearly established, our results demonstrated that high values of disaturated PG are not necessary. Rather, the observed wide spectrum of PG fatty acid compositions suggests that the acidic head group may be of more importance than the acyl chain composition.

The differences in surfactant phospholipid fatty acid composition may reflect to some extent differences in the metabolic pathways

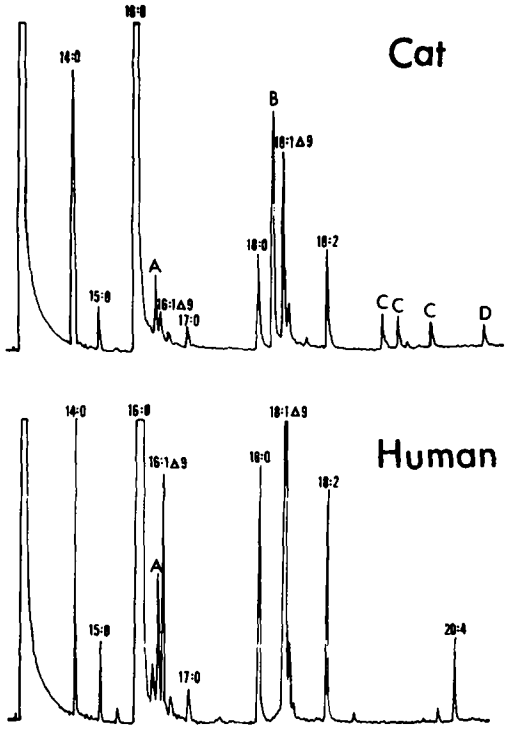

FIG. 1. Chromatograms of cat and human surfactant PC fatty acid methyl esters separated on Silar 10C glass capillary column. A, unusual monounsaturated 16-carbon fatty acid, 16:1Δ7. Two additional peaks of 16-carbon fatty acids can be seen clearly in the human surfactant chromatogram, one before A and the other after 16:1Δ9. These peaks are identified tentatively as 16:1Δ5 and 16:1Δ11 on the basis of their relative retention times. B, 18:1Δ5; C, 20-carbon fatty acid methyl esters, and D, 22-carbon fatty acid methyl ester. Note the absence of arachidonic acid (20:4) in cat surfactant PC.

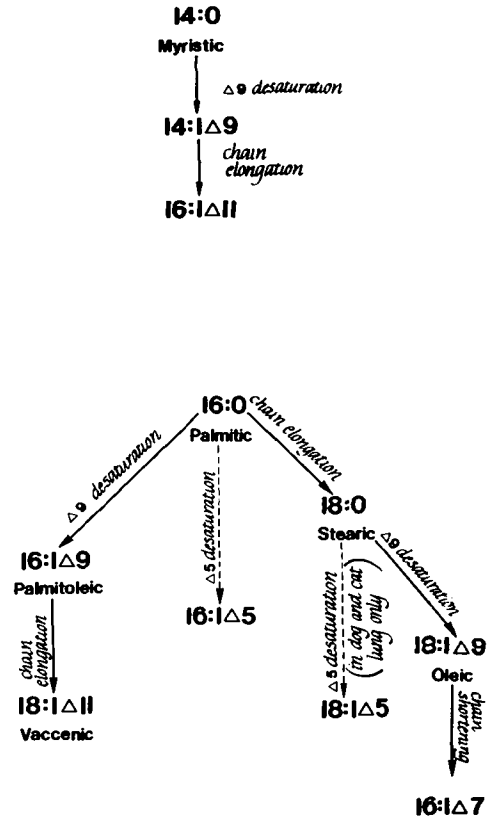

FIG. 2. Diagrammatic representation of possible pathways for formation of monounsaturated fatty acids found in lung surfactant phospholipids.

TABLE 4

Hexadecenoic Acids of Surfactant Phospholipids – Ratio of Palmitoleic Acid, 16:1Δ9, to 16:1Δ7

	Human	Cat	Dog	Rabbit	Rat
Phosphatidylcholine	2.4	0.3	0.3	2.6	0.9
Phosphatidylglycerol	6.8	0.3	0.4	7.0	Not Done

and their regulation in different species. The presence of 18:1Δ5 suggests the existence of a unique enzymatic activity in cat and dog lung (Fig. 2), since Δ5 fatty acyl CoA desaturase normally uses unsaturated fatty acyl CoA as substrate (19). By contrast, variable amounts of the unusual 16:1 isomer are present in all species studied. This fatty acid does not appear to be specific for surfactant but is more actively incorporated into PC, as compared to PG, in humans and rabbits than in cats and dogs, where the ratio of 16:1Δ9/16:1Δ7 is the same in both PC and PG. The presence of several unusual fatty acids in cat lung surfactant also

is most likely a reflection of overall lipid metabolic pathways. Cats, unlike the other species studied, are unable to synthesize arachidonic acid from linoleic acid (20) and appear to use other pathways resulting in the formation of unusual fatty acids which are available for incorporation into surfactant phospholipids. These considerations as well as the differences among animal species in percentages of monounsaturated and polyunsaturated fatty acids and total saturated to unsaturated fatty acid ratios suggest that a wide variety of molecular species of PC and PG are suitable for surfactant phospholipids, provided that a certain minimum quantity of DPPC is maintained.

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REFERENCES

1. Pattle, R.E. (1955) Nature (London) 175, 1125-1126.
2. Clements, J.A. (1957) Proc. Soc. Exp. Biol. Med. 95, 170-172.
3. Avery, M.E. and Mead, J. (1959) Am. J. Dis. Child. 97, 517-523.
4. Sanders, R.L. (1982) in Lung Development: Biological and Clinical Perspectives (Farrell, P.M., ed.) Vol. 1, pp. 193-210, Academic Press, New York.
5. Sanders, R.L. (1982) in Lung Development: Biological and Clinical Perspectives (Farrell, P.M., ed.) Vol. 1, pp. 211-219, Academic Press, New York.
6. Shelley, S.A., Balis, J.U., Paciga, J.E., Espinoza, C.G., and Richman, A.V. (1982) Lung 160, 195-206.
7. Shelley, S.A., Paciga, J.E., and Balis, J.U. (1981) Proc. Fed. Am. Soc. Exp. Biol. 40, 796.
8. Paciga, J.E., Shelley, S.A., and Balis, J.U. (1980) Biochim. Biophys. Acta 631, 487-494.
9. Shelley, S.A., Paciga, J.E., and Balis, J.U. (1977) Lipids 12, 505-510.
10. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497-509.
11. Wuthier, R.E. (1966) J. Lipid Res. 7, 544-550.
12. Vaskovsky, V.E., Kostetsky, E.Y., and Vasendin, I.M. (1975) J. Chromatogr. 114, 129-141.
13. Muneshige, A., Okazaki, T., Quirk, J.G., MacDonald, P.C., Nozaki, M., and Johnston, J.M. (1983) Am. J. Obstet. Gynecol. 145, 474-480.
14. Sun, G.Y., and Horrocks, L.A. (1970) Lipids 5, 1006-1012.
15. Stein, R.A., and Nicolaides, N. (1962) J. Lipid Res. 3, 476-478.
16. King, R.J., Ruch, J., and Clements, J.A. (1973) J. Appl. Physiol. 35, 778-781.
17. Schürch, S., Goerke, J., and Clements, J.A. (1976) Proc. Nat. Acad. Sci. U.S.A. 73, 4698-4702.
18. Shelley, S.A., Kovacevic, M., Paciga, J.E., and Balis, J.U. (1979) N. Engl. J. Med. 300, 112-116.
19. Jeffcoat, R. (1979) Essays Biochem. 15, 1-36.
20. Rivers, J.P.W., Sinclair, A.J., and Crawford, M.A. (1975) Nature (London) 258, 171-173.

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Peroxidation of Free and Esterified Fatty Acids by Horseradish Peroxidase

CHARLES W. GARNER, *Texas Tech University Health Sciences Center, Department of Biochemistry, Lubbock, TX 79430*

ABSTRACT

Linoleic and arachidonic acids and unsaturated esterified fatty acids of soybean lecithin liposomes were oxidized by horseradish peroxidase in the presence of hydrogen peroxide. The major products formed in the presence of oxygen were fatty acid hydroperoxides. In the absence of oxygen, other unidentified products were formed. Diene conjugation was about 5 times faster in oxygen than in nitrogen. Malondialdehyde was formed only in the presence of oxygen. Superoxide, singlet oxygen and hydroxyl radical may have been involved in the free fatty acid oxidation system but not in the liposome system. Replacement of hydrogen peroxide with the hydrogen peroxide (and superoxide) generators xanthine oxidase or galactose oxidase caused a more efficient oxidation in the presence of peroxidase than in its absence, suggesting that the in vivo toxicity of hydrogen peroxide and superoxide may be greatly increased in the presence of peroxidase.

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INTRODUCTION

Lipid peroxidation proceeds through a free radical mechanism resulting in the formation of mainly fatty acid hydroperoxides but also lesser amounts of carbonyls and hydrocarbons. Many aspects of this process have been studied in detail and have been reviewed (1-6). Peroxidation of lipids is not entirely a deleterious event, since some normal processes appear to involve lipid peroxides. These include prostaglandin synthesis (7), phagocytosis (8) and aging (9). However, it is clear that an uncontrolled peroxidative attack on a cell would result in its demise.

The expiration of hydrocarbons demonstrates that lipid peroxidation occurs in vivo (10). However, many problems remain, such as determining how much peroxidation should be considered normal and beneficial, determining what are the roles of activated oxygen species (i.e. superoxide, hydrogen peroxide and hydroxyl radical) and determining what enzyme systems are responsible for lipid peroxidation.

In this report, it is shown that horseradish peroxidase catalyzes the peroxidation of fatty acids in the presence of very small amounts of hydrogen peroxide or superoxide. It is suggested that several important functions of peroxidases may be mediated through lipid peroxidation.

MATERIALS AND METHODS

Horseradish peroxidase type VI (donor: H_2O_2 oxidoreductase, EC 1.11.1.7), superoxide dismutase Type I, xanthine oxidase Grade III, galactase oxidase Type V, mannitol, histidine, sodium taurodeoxycholate, soybean lecithin, dicetyl phosphate, cholesterol, thiobarbituric acid, diethylenetriaminepentacetic acid (DTPA) and EDTA were purchased from Sigma Chemical Co., St. Louis, Missouri. Arachidonic and linoleic acids were products of Nu Chek Prep, Inc., Elysian Field, Minnesota. Tetramethoxypropane was from Aldrich Chemical Co. Hydrogen peroxide and *t*-butyl alcohol were from products of MCB Reagents, Cincinnati, Ohio. 2,6-Di-*t*-butyl-4-hydroxymethyl phenol was from K and K Rare and Fine Chemicals, Plainview, New York. Thin layer plates were 20 × 20 cm glass plates with 200 μm layers of silica gel from Analtech, Newark, Delaware. Other reagents used were purchased from usual sources as the highest grade available. Glass distilled deionized water was used in all solutions.

Fatty acid solution. An optically clear solution of linoleic or arachidonic acid was prepared using taurodeoxycholate as solubizer. Fatty acid, 12 μmol, was added to a test tube and the solvent was removed in a stream of nitrogen. Taurodeoxycholate, 5.5 ml of 55 mM, was added. The suspension was sonicated under nitrogen for 5 min until optically clear. The solution was then increased in volume to 20 ml and contained in addition 0.45 M NaCl, 0.15 M potassium phosphate, pH 6.2, and 3 mM EDTA. The solution was then filtered through a nitrocellulose filter, HAWP (Millipore). The solution could be stored at 4 C or at -20 C for a month with little oxidation. Reaction mixtures contained 0.2 ml of this solution in a final volume of 0.6 ml. Taurodeoxycholate was inhibitory at concentrations above 8 mM.

Liposome solution. Liposomes were prepared by injecting a 1-ml ethanol solution of soybean lecithin, 5 μmol; dicetyl phosphate, 2.5 μmol; and cholesterol, 2.5 μmol, into 50 ml

of buffer containing 0.15 M NaCl, 1 mM EDTA and 0.05 M potassium phosphate at pH 7.2. The final reaction mixture of 0.6 ml contained 0.1 ml of this solution in the same buffer. The liposome mixture was stored frozen. No effect of freezing of liposomes on substrate activity was noted.

Measurement of diene conjugation. Diene conjugation was measured from the change in absorbance at 233 nm in a Beckman Acta M VI UV/Vis Spectrophotometer at 35 C ± 0.1 C. An extinction coefficient of 25,000 $M^{-1} cm^{-1}$ (11) was assumed.

Thin layer chromatography of fatty acid peroxides. Appropriate reaction mixtures were made 0.1 M in HCl and extracted two times with 5 ml of ethyl ether. The ether layers were combined and reduced to dryness under a stream of nitrogen. The remaining volatile solvents were removed in vacuo. The residue was dissolved in 0.1 ml of chloroform. Aliquots containing 14 μg of fatty acid were spotted onto the chromatography plate. The plate was developed with ether:hexane:acetic acid (50:50:1, v/v/v) and was stained in iodine vapor or with a starch-iodine spray (12).

Malondialdehyde determination. Malondialdehyde was measured by the thiobarbituric acid procedure of Dahle et al. (13) using tetramethoxypropane as a standard.

Oxygen measurements. Oxygen consumption was measured with an oxygen electrode (Yellow Springs Instrument Company, Inc., Yellow Springs, Ohio) in a reaction mixture of 1.2 ml using an appropriate recorder. Absorption measurements made under pure oxygen or nitrogen were made in an anerobic cuvette.

RESULTS

Horseradish peroxidase catalyzed the peroxidation of linoleic and arachidonic acids. This could be followed by the absorbance change at 233 nm due to the conjugation of double bonds. As shown in Table 1, the reaction was dependent on the presence of peroxidase and hydrogen peroxide. The reaction was inhibited by a variety of antioxidants and peroxidase substrates. Denaturation of the enzyme by heating destroyed 90 to 95% of the activity. Various scavengers of activated oxygen species such as the superoxide scavenger superoxide dismutase, the hydroxyl radical scavengers mannitol (14) and *t*-butyl alcohol (15), and the singlet oxygen scavengers histidine (16-18) and uric acid (30) were inhibitory only in the free fatty acid system. Differences in the solubility of these inhibitors in micelles and in liposomes might

TABLE 1

Lipid Peroxidation as Measured by Diene Conjugation

Reaction mixture	ΔA_{233} (% control)	
	Linoleic acid	Liposomes
complete[a]	(100)	(100)
– peroxidase	0	0
– hydrogen peroxide	1	0
– EDTA	64	92
– EDTA + DTPA (1.7 mM)	58	105
complete with heat-treated peroxidase[b]	4	11
+ antioxidant[c]	0	0
+ superoxide dismutase, 1.0 μg	38	95
+ *tert*-butyl alcohol, 75 mM	33	89
+ mannitol, 0.177 M	21	92
+ histidine, 60 mM	61	103
+ uric acid, 2 μM	50	94
+ catalase, 1 μg	2	5

[a]The complete fatty acid reaction mixture contained in a volume of 0.6 ml, 0.05 M potassium phosphate, pH 6.2; 0.15 M NaCl; 5 mM taurodeoxycholate; 1 mM EDTA; 0.2 mM linoleic acid; 0.17 mM hydrogen peroxide and 1.0 μg peroxidase. Identical results were obtained with arachidonic acid. The control rate was 0.0055 A_{233} units per min. The complete liposome reaction mixture contained in a volume of 0.6 ml, 0.05 M potassium phosphate, pH 7.2; 0.15 M NaCl; 1 mM EDTA; 0.17 mM hydrogen peroxide; 1.0 μg peroxidase and liposomes prepared as described in the methods section. The control rate was 0.0091 A_{233} units per min.

[b]Peroxidase heated at 88 C for 10 min.

[c]0.17 mM 2,6-di-*tert*-butyl-4-hydroxymethyl phenol.

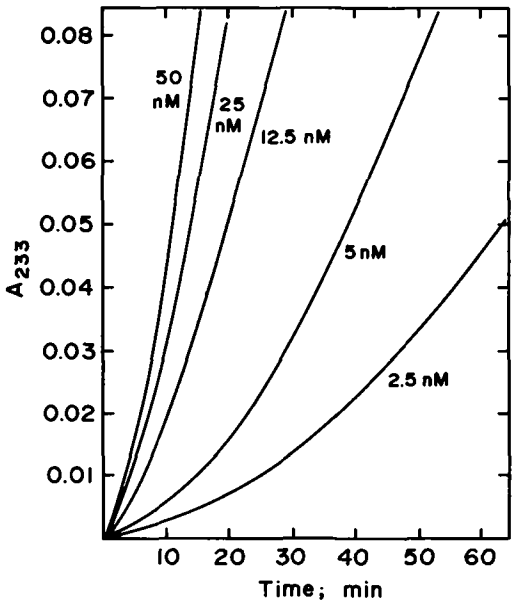

FIG. 1. Peroxidation of linoleic acid with different amounts of peroxidase. The reaction mixture described in the first footnote to Table 1 was supplemented with peroxidase in the amounts indicated beside each line in the figure. Each mixture contained linoleic acid.

account for differences in their action. Omitting the NaCl or increasing the NaCl concentration to 0.5 M had no effect on oxidation in either system.

Increasing the amount of peroxidase increased the rate of oxidation. This is shown in Figure 1 with linoleic acid as the substrate. The reactions with linoleic acid or with soybean lecithin liposomes were autocatalytic as shown by the increase in slope of each curve with time. After an increase in absorbance of about 0.04, the curves became linear. Reaction rates were estimated after the absorbance change became linear. Reactions proceeded to about 30 to 50% completion assuming a stoichiometric conversion of hydrogen peroxide to conjugated diene. Reactions with arachidonic acid as substrate (data not shown) also were nonlinear, with the reaction rates decreasing with time instead of increasing. Reaction rates were measured at zero time with arachidonic acid as substrate.

The reaction rate was nearly constant at hydrogen peroxide concentrations between 1 μM and 0.1 mM (Fig. 2). High concentrations of hydrogen peroxide were inhibitory, while very low concentrations gave reduced rates of oxidation. The measurement of oxidation rates at hydrogen peroxide concentrations below 0.2 μM were limited by the availability of substrate.

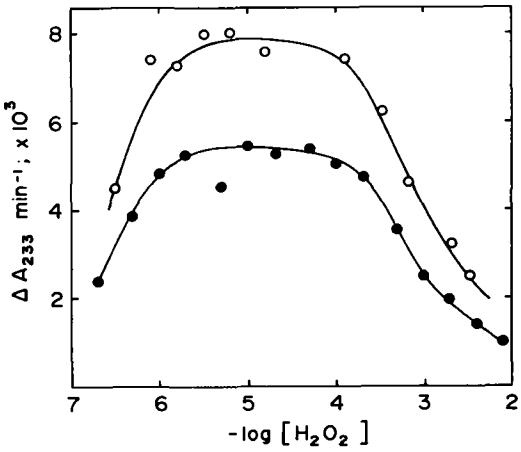

FIG. 2. Peroxidation of fatty acids and liposomes at different concentrations of hydrogen peroxide. The reaction mixtures are described in the first footnote to Table 1. (—•—) linoleic acid. (—o—) liposomes.

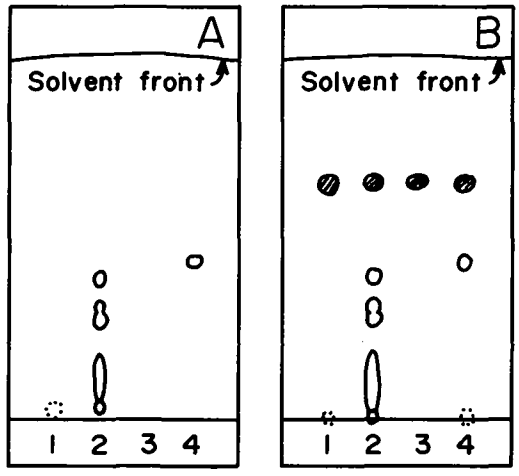

FIG. 3. Thin layer chromatography of peroxidized fatty acids. The reaction mixture described in the Table 1 footnotes contained arachidonic acid (lanes 1 and 2) or linoleic acid (lanes 3 and 4) and was incubated for 16 hr at 35 C. Reaction mixtures spotted onto lanes 1 and 3 contained no peroxidase. The fatty acids were extracted and chromatographed as described in the Materials and Methods Section. Plate A was sprayed with starch-iodide to stain hydroperoxides. Plate B was stained in iodine vapor to stain all lipid components.

The increase in absorbance at 233 nM was presumed to be due to the accumulation of fatty acid dienes predominantly as hydroperoxides. The presence of hydroperoxides was demonstrated by thin layer chromatography (TLC), shown in Figure 3. Iodine vapor was used to stain all of the lipid components in an ether extract of a typical reaction mixture,

TABLE 2

Effect of Oxygen and Nitrogen on Linoleic Acid Peroxidation[a]

Gas	Malondialdehyde[b] μM	1,3-diene[c] μM
N_2	0.3	14
O_2	3.2	69

[a]The reaction mixture, described in footnote 1 to Table 1, was incubated under the gas indicated at 37 C for 16 hr.

[b]Measured by the thiobarbituric acid test.

[c]Measured from the absorption at 233 nm.

while a starch-iodine spray (12) was used to stain only fatty acid hydroperoxides. Multiple peroxide products were seen with arachidonic acid, while only a single major spot was seen with linoleic acid. Hydroperoxide formation was dependent upon the presence of peroxidase, hydrogen peroxide and oxygen.

Replacing the air atmosphere above the reaction mixture with pure oxygen increased reaction rates. When the reaction mixture was under nitrogen the reaction rates were diminished, but they did not reach zero. Typically a 5-fold difference in reaction rate was seen between the use of oxygen and nitrogen atmospheres. In the experiment with free acids shown in Table 2, 69 μM conjugated diene accumulated during a 16-hr incubation under oxygen, while only 14 μM conjugated diene accumulated under nitrogen. The major species formed in the presence of oxygen was apparently a fatty acid hydroperoxide which was detected by reaction with thiobarbituric acid (13). Hydroperoxides were evident when the reaction was carried out in the presence of oxygen, while in the presence of nitrogen, very little hydroperoxide was formed. The formation of conjugated dienes in the absence of oxygen apparently represents the formation of species other than hydroperoxides. Oxygen was consumed during the reaction.

Since the stimulation of reaction rates in the presence of oxygen might have been due to reaction with activated oxygen species, several scavengers of these species were tested as inhibitors of the oxidation in air as measured by the change in absorbance at 233 nm. As noted in Table 1, the superoxide scavenger, superoxide dismutase, inhibited peroxidation of free fatty acids. Upon the addition of increasing amounts of superoxide dismutase, inhibition increased to a maximum of about 70 to 80% in air (Fig. 4). In the presence of nitrogen, the inhibition reached only 9%. An involvement of the hydroxyl radical was suggested by the inhibition of *t*-butyl alcohol and mannitol, known hydroxyl radical scavengers (14,15). With increasing amounts of mannitol (or *t*-butyl alcohol), a maximum of about 80% inhibition was attained. Singlet oxygen may have been involved since histidine (16-18) when added in increasing amounts inhibited about 40%. Similar data were obtained with uric acid (not shown) with maximum inhibition reaching about 58% at a concentration of about 30 μM. As seen in Table 1, omission of EDTA reduced the rate of oxidation by 33%. DTPA but not EDTA inactivates trace heavy metals (19). This suggests some role of heavy metal contaminants in the reaction with detergent-stabilized solutions of fatty acids, but not in liposomes.

Oxidation of fatty acids by peroxidase occurred at very low concentrations of hydrogen peroxide. Hydrogen peroxide at 200 nM allowed diene conjugation to proceed at an

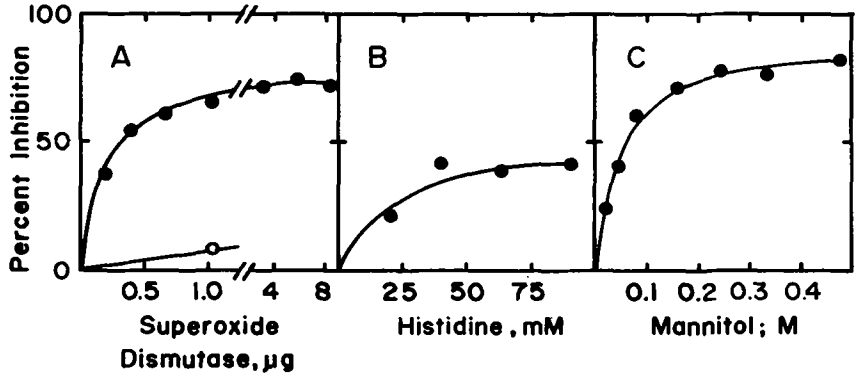

FIG. 4. Inhibition of peroxidation by oxygen scavengers. The reaction mixtures contained linoleic acid and are described in the first footnote to Table 1. A. Inhibition by superoxide dismutase in the presence of air (--•--) or nitrogen (—o—). B. Inhibition by histidine. C. Inhibition by mannitol.

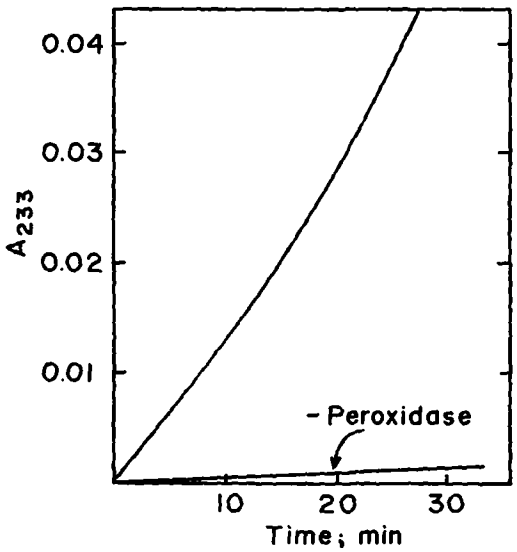

FIG. 5. Peroxidation of linoleic acid by peroxidase with galactose/galactose oxidase in place of hydrogen peroxide. The reaction mixture described in the first footnote to Table 1, minus hydrogen peroxide, was supplemented with 1 unit of galactose oxidase and 10 mM galactose and contained linoleic acid.

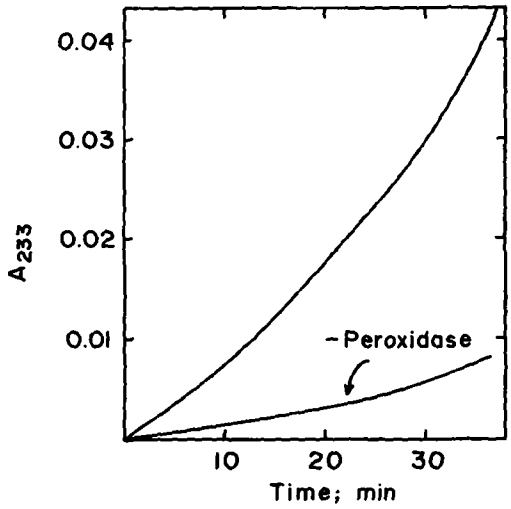

FIG. 6. Peroxidation of linoleic acid by peroxidase with acetaldehyde/xanthine oxidase in place of hydrogen peroxide. The reaction mixture described in the first footnote to Table 1, minus hydrogen peroxide, was supplemented with 0.3 μg of xanthine oxidase and 30 mM acetaldehyde and contained linoleic acid.

appreciable rate, although the extent of conversion was limited by the availability of hydrogen peroxide. At very low concentrations of hydrogen peroxide, 1 mol of diene was formed for each mol of hydrogen peroxide consumed.

It was expected that the low concentrations of hydrogen peroxide produced by certain oxidative enzyme systems would be sufficient to cause lipid peroxidation in the presence of peroxidase. This was observed readily with both fatty acids or liposomes as substrates. In Figure 5 the oxidation of linoleic acid by peroxidase in the presence of galactose and galactose oxidase is described. The rates obtained with this system were similar to those obtained with exogenously added hydrogen peroxide. In the absence of peroxidase there was a negligible rate of oxidation. In the presence of xanthine oxidase and acetaldehyde, shown in Figure 6, peroxidase again catalyzed linoleic acid oxidation. With this system there was a low rate of oxidation in the absence of peroxidase, presumably due to the production of superoxide by this system. Thus, peroxidase potentiates the oxidative capacity of superoxide and hydrogen peroxide.

DISCUSSION

Because of the potent cytotoxicity properties of certain peroxidases (20), I have investigated the peroxidation of lipids by horseradish peroxidase, a peroxidase which is available commercially in pure form and which is itself cytotoxic (unpublished results), in order to determine if membrane damage is possibly the mechanism of cell destruction. Lipid peroxidation is well known to cause membrane damage and cell death (21).

Horseradish peroxidase was found to mount an aerobic attack on fatty acids bearing 2 or more double bonds either in detergent solution or in liposomes. In the presence of oxygen, hydroperoxides were formed as indicated by oxygen consumption, malondialdehyde formation and diene conjugation. In the absence of oxygen, diene conjugation also was observed, although at a reduced rate. These observations are consistent with a free-radical mechanism in which peroxidase in the presence of hydrogen peroxide catalyzes the abstraction of a hydrogen atom from the methylene group between 2 double bonds. The resulting lipid radical in turn reacts accordingly with other lipids, oxygen, etc.

The mechanism of how peroxidase can catalyze the abstraction of a hydrogen atom is unknown, although it appears that the formation of activated oxygen species by peroxidase is not required. Oxidation of the lipid is presumed to have occurred via a direct interaction of the enzyme with the lipid substrate.

The lack of apparent involvement of activated oxygen species in the peroxidation of liposomes

was possibly the result of the significantly higher rate of peroxidation. At pH 7.2 the rate of oxidation of liposomes was 5-10 times faster than the rate of oxidation of free fatty acids in detergent micelles. The meaning of this difference in rate is obscured by the difference in the physical states of the substrates.

The ratio of bile salt to fatty acid was 25, which is approximately equal to the aggregation number of taurodeoxycholate under these conditions (22). This might have lead to the formation of activated oxygen species as a side reaction which in turn also participated in lipid oxidation.

This peroxidase has some different properties from other often studied peroxidases, such as myeloperoxidase and lactoperoxidase. However, these observations are extended readily to biological systems. For example, rat uterine peroxidase has been shown (unpublished results) to catalyze the peroxidation of lipids in the same systems used in this study. Recently Kanner and Kinsella (23) demonstrated the peroxidation of lipids by lactoperoxidase.

Several animal cell organelles such as mitochondria (24), microsomes (25) and nuclei (26) produce hydrogen peroxide. Hydrogen peroxide may have some physiological significance in metabolic regulation (27,28), even though scavengers of hydrogen peroxide like catalase and glutathione peroxidase are normally present. Also, peroxidases are found in many tissues (29). Thus, the potential for oxidative damage such as that described in this report exists during periods of hydrogen peroxide production. As was demonstrated in this work, sub-micromolar amounts are sufficient to cause peroxidation. Since peroxidases have such low specificity for the hydrogen donor, the possibility exists that the role of peroxidases in tissues may be other than that classically proposed, namely the removal of hydrogen peroxide. It is possible that lipid peroxidation may serve a role in the mediation of hormonal effects, in protection from microbial attack or in normal cell death.

ACKNOWLEDGMENT

Michelle Durham provided technical assistance and Harvey Olney provided art work. This work was aided by grant #IN-148 from the Americal Chemical Society.

REFERENCES

1. Tappel, A.L. (1973) Federation Proc. 32, 1870-1874.
2. Tappel, A.L. (1980) in Free Radicals in Biology (W.A. Pryor, ed.) Vol. 4, pp. 2-47, Academic Press, New York.
3. Vladimirov, Y.-A., Olenov, V.I., Suslova, T.B., and Cheremisina, T.P. (1980) Adv. Lipid Res. 17, 173-249.
4. Mead, J.F. (1976) in Free Radicals in Biology (W.A. Pryor, ed.) Vol. 1, pp. 51-68, Academic Press, New York.
5. McCay, P.B., and Poyer, J.L. (1976) in The Enzymes of Biological Membranes, (A. Martonosi, ed.) Vol. 4, pp. 239-256, New York, NY.
6. Recknagel, R.O., and Glende, E.A. (1977) in Reactions of Environmental Agents, (D.H.K. Lee, ed.), Section 9, pp. 591-601. Am. Physiol. Soc., Bethesda, MD.
7. Samuelsson, B., Granstrom, E., Green, K., Hamberg, M., and Hammarstrom, S. (1975) Annu. Rev. Biochem. 44, 669-695.
8. Klebanoff, S.J. (1975) Semin. Hematol. 12, 117-142.
9. Barber, A.A., and Bernheim, F. (1967) Adv. Gerontol. Res. 2, 355-403.
10. Tappel, A.L., and Dillard, C.J. (1981) Federation Proc. 40, 174-178.
11. Johnston, A.E., Zilch, K.T.,, Selke, E., and Dutton, H.J. (1961) J. Am. Oil Chem. Soc. 38, 367-371.
12. Vioque, E., and Holman, R.T. (1962) Arch. Biochem. Biophys. 99, 522-528.
13. Dahle, L.K., Hill, E.G., and Holman, R.T. (1962) Arch. Biochem. Biophys. 98, 253-261.
14. Dorfman, L.M., and Adams, G.E. (1973) Reactivity of the Hydroxyl Radical in Aqeous Solutions, pp. 1-59, NSRDS-NBS No. 46, United States Department of Commerce, National Bureau of Standards.
15. Kellogg, E.W., and Friedovich, I. (1975) J. Biol. Chem. 250, 8812-8817.
16. Wasserman, H.H. (1970) Ann. N.Y. Acad. Sci. 171, 108-119.
17. Tomita, M., Irie, M., and Ukita, Ta. (1969) Biochemistry 8, 5149-5160.
18. Wasserman, H.H., Stiller, K., and Floyd, M.B. (1968) Tetrahedron Lett. 29, 3277-3280.
19. Halliwell, B. (1978) FEBS Lett. 92, 321-326.
20. Klenbanoff, S.J., and Clark, R.A. (1975) Blood 45, 699-707.
21. Pryor, W.A. (1980) in Autoxidation in Food and Biological Systems (Simic, M.G. and Karel, M., eds.) p. 1,, Plenum Press, New York, NY.
22. Small, D.M. (1968) in Molecular Association in Biological and Related Systems (Goddard, E.D., ed.) p. 31, American Chemical Society, Washington, D.C.
23. Kanner, J., and Kinsella, J.E. (1983) Lipids 18, 204-210.
24. Boveris, A., and Chance, B. (1973) Biochem. J. 134, 707-716.
25. Hildebrandt, A.G., and Roots, I. (1975) Arch. Biochem. Biophys. 171, 385-397.
26. Bartoli, G.M., Galeotta, T., and Ozzi, A. (1977) Biochim. Biophys. Acta 497, 622-626.
27. Mukherjee, S.P. (1980) Biochem. Pharmacol. 29, 1239-1246.
28. May, J.M., and de Haen, C. (1979) J. Biol. Chem. 254, 2214-2220.
29. Neufeld, H.A., Levay, A.N., Lucas, F.V., Martin, A.P., and Statz, E. (1958) J. Biol. Chem. 233, 209-211.
30. Ames, B.N., Cathcart, R., Schwiers, E., and Hochstein, P. (1981) Proc. Natl. Acad. Sci. 78, 6858-6862.

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The Effects of *trans* Fatty Acids on Fatty Acyl Δ5 Desaturation by Human Skin Fibroblasts

MIRIAM D. ROSENTHAL* and **MARK A. DOLORESCO**, *Department of Biochemistry, Eastern Virginia Medical School, Norfolk, VA 23501*

ABSTRACT

The effectiveness of different fatty acids as inhibitors of fatty acyl Δ5 desaturation activity in human skin fibroblasts has been investigated. When incubated with 2.25 μM [^{14}C] eicosatrienoate (20:3ω6) in otherwise lipid-free medium, these cells rapidly incorporate the radiolabeled fatty acid into cellular glycerolipids and desaturate it to produce both [^{14}C]arachidonate and [^{14}C]docosatetraenoate. The Δ5 desaturation activity can be enhanced by prior growth of the cells without serum lipids. Elaidate (9t-18:1) is a potent inhibitor of Δ5 desaturation while *trans*-vaccenate (11t-18:1) is virtually without effect. Oleate and linoleate are only mildly inhibitory. Linoelaidate (9t,12t-18:2) is more inhibitory than linoleate but significantly less effective than elaidate. The effects of elaidate can be readily overcome by increasing the concentration of exogenous eicosatrienoate. Studies with a variety of *trans* monounsaturates of differing chain lengths indicate that the ω9 *trans* fatty acids are potent inhibitors of Δ5 desaturation, while ω7 *trans* fatty acids are relatively ineffective. Intact human fibroblasts could thus be important in characterizing novel fatty acids as selective inhibitors of arachidonate synthesis in vivo.

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INTRODUCTION

Mammalian cells contain a series of microsomal enzymes which catalyze the desaturation and elongation of dietary linoleate (9c,12c-18:2) to produce longer chain 20- and 22-carbon polyunsaturated fatty acids. Fatty acyl Δ5 desaturase is of special interest in that it converts the CoA ester of 8c,11c,14c-eicosatrienoate to arachidonate (5c,8c,11c,14c-eicosatetraenoate) (1). In addition to being major components of cellular phospholipids, these two fatty acids are the precursors of the 1- and 2-series prostaglandins (2). In most tissues arachidonate normally predominates over 20:3 as a component of membrane phospholipids (3) and as a substrate for the cyclooxygenase and lipoxygenase enzymes. Inhibition of fatty acyl Δ5 desaturation could thus shift the synthesis of prostaglandins, resulting in an increase in PG_1's relative to PG_2's (4). Regulation of enzyme activity may be of particular importance in humans, where the level of Δ5 desaturase activity appears to be less than in the mouse or rat (5).

The mixtures of *trans* fatty acids present in partially hydrogenated vegetable oils have been shown to suppress synthesis of arachidonic acid in vivo and to exacerbate symptoms of essential fatty acid deficiency (6,7). Although in vivo studies have demonstrated inhibition of the initial Δ6 desaturation of linoleate by *trans* monounsaturates (8) and linoelaidate (9t,12t-18:2) (9), they provide only indirect data on Δ5 desaturation activity. In vitro studies using liver microsomal preparations have shown that a variety of isomeric *cis*- and *trans*-octadecenoic acids directly inhibit the desaturation of 8,11,14-eicosatrienoyl CoA (10,11)

Our laboratory has developed a protocol for investigating fatty acyl desaturation activities in intact human cells (12). Human skin fibroblasts readily incorporate exogenous free fatty acids into cellular phospholipids and triacylglycerol, thus facilitating nutritional modification of cellular composition. We have shown that both linoelaidate and elaidate are potent inhibitors of Δ6 desaturation in these cells. By contrast, the Δ9 desaturation of [^{14}C]stearate is inhibited by *cis* but not by *trans* fatty acids. Interestingly, the relative effectiveness of various fatty acids in the intact cell system is different from that found in microsomal studies. The cell culture system thus appears to be a useful tool for evaluating the physiological effects of isomeric fatty acids on cellular metabolic processes.

The present study uses this cell culture system to examine fatty acyl Δ5 desaturation in human cells. In particular we have investigated the effects of medium supplementation with individual *trans* fatty acids on the synthesis of arachidonate from [^{14}C]eicosatrienoate.

MATERIALS AND METHODS

Cell Culture

Normal human skin fibroblasts derived from a 3-month fetus (GM-10) were obtained from the NIGMS Human Genetic Mutant Cell Repos-

*To whom correspondence should be addressed.

itory (Camden, New Jersey). Cells were propagated in Eagle's Minimum Essential Medium containing 10% Fetal Bovine Serum (Gibco, Grand Island, New York) as described previously (12,13). Each experiment used replicate flasks of subconfluent, actively mitotic cells with approximately 600 μg cellular protein/25cm^2 flask. Delipidized calf serum was prepared by acetone:ethanol (1:1 v/v) extraction (14) and reconstituted in Earle's Balanced Salt Solution. The delipidized serum was used to replace complete serum for 4 days prior to each experiment as well as during fatty acid supplementation.

Fatty Acid Supplementation

(8c,11c,14c)-[1-^{14}C]Eicosatrienoate (54.9 mCi/mmol) was obtained from New England Nuclear Corp. (Boston, Massachusetts). The *trans* fatty acids 9t-16:1, 10t-17:1, 10t-19:1 and 11t-20:1 were obtained from NuChek Prep, Inc. (Elysian, Minnesota), and other fatty acids were from Sigma (St. Louis, Missouri). The fatty acids were stored in hexane at -20 C under a nitrogen atmosphere. Concentrations and purity of the free fatty acids were confirmed by gas liquid chromatography (GLC) after methylation with BCl_3 in methanol using internal standards (12). For each experiment, aliquots of fatty acid solutions were evaporated to dryness under N_2 and resuspended in 95% EtOH. The fatty acids were then transferred quantitatively to reconstituted delipidized serum and diluted with culture medium (15).

Lipid Extraction and Analysis

The cells were harvested by trypsinization and cellular lipids extracted in a 2:1:1 mixture of ethyl acetate/acetone/cell suspension (16). Fatty acid methyl esters were prepared from cellular lipids using methanolic base. Gas-liquid chromatography was performed on a Packard 427 chromatograph (Downers Grove, Illinois) with flame ionization detection using a 6-ft glass column packed with 10% SP-2330 on 100/120 Chromasorb W AW (Supelco, Bellefonte, Pennsylvania). After a 3 min initial hold, the oven was programmed from 160 to 235 C at 2 degrees/min. The distribution of [^{14}C] fatty acid methyl esters was determined with a Packard 894 Gas Flow Proportional Counter interfaced to the chromatograph. A Linear dual-pen recorder was used to obtain simultaneous radioactivity and mass tracings of each chromatographic separation; a Spectra-Physics (Santa Clara, California) Minigrator was used to integrate radioactivity peak areas. Thin layer chromatography (TLC) to separate neutral lipids and the major classes of phospholipids was performed as described previously (13).

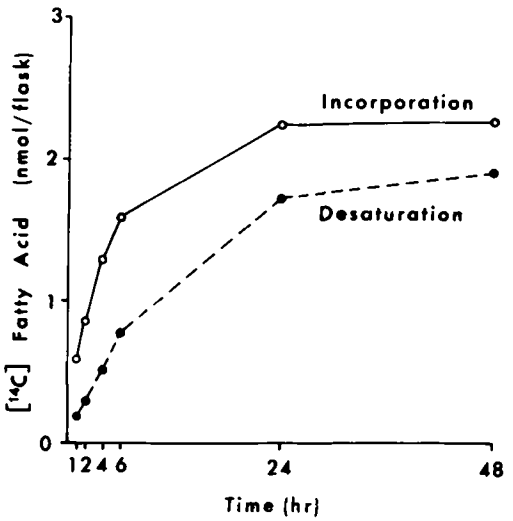

FIG. 1. Time course of incorporation of [1-^{14}C] eicosatrienoate into cellular lipids and its desaturation to arachidonate plus docosatetraenoate. GM-10 cells were grown for 4 days in medium without serum lipids. They were then incubated with 0.25 μCi [^{14}C] eicosatrienoate in 4ml Eagle's minimum essential medium plus delipidized serum protein as described in Materials and Methods. At each time point, 2 replicate flasks were harvested and cellular lipids analyzed. o – o, total incorporation into cellular lipids, of which >96% is glycerolipids; • - - •, percentage desaturation calculated from [^{14}C] arachidonate plus [^{14}C] docosatetetraenoate (22:4) divided by total cellular ^{14}C-fatty acids.

RESULTS AND DISCUSSION

Figure 1 shows the results obtained when subconfluent, actively mitotic human skin fibroblasts are incubated with 2.3 μM [1-^{14}C] eicosatrienoate in medium with delipidized serum. Initial incorporation of the exogenous [^{14}C] 20:3 into cellular lipids is quite rapid, reaching 48% in 6 hr. Active desaturation of the incorporated [^{14}C] 20:3 is seen; within 1 hr 33% of the cellular ^{14}C-acyl groups consist of arachidonate (20:4) plus docosatrienoate (22:4). Δ5 desaturation activity continues after incorporation has slowed considerably, and the percentage desaturated rises to 85% in 48 hr. Throughout this process, most of the radioactivity in cellular lipids was in phospholipids. For example, at 6 hr 86.8% of the cellular ^{14}C-acyl groups were esterified in phospholipids, with 9.5% in triacylglycerol and 3.0% in diacylglycerol.

The data shown in Figure 1 was obtained with cells which had been grown for 4 days in lipid-free medium prior to the experiment. A comparison was made between these cells and replicate flasks grown with 10% fetal bovine serum. The incubations with [^{14}C] 20:3 were performed under identical conditions. Although

total incorporation at 6 and 24 hr was similar for both groups, the cells grown with fetal bovine serum exhibited 40% less desaturation of the incorporated [^{14}C]20:3 (data not shown). Previous studies in our laboratory (12) have demonstrated a similar enhancement of both fatty acyl Δ9 and Δ6 desaturation activities in human skin fibroblasts after growth without serum lipids. Regulation of fatty acid synthesis and fatty acyl Δ9 and Δ6 desaturation in fibroblasts are thus similar to what is observed in vivo. Dietary regulation of Δ5 desaturase activity in vivo has not, however, been completely elucidated. One recent study (17) found that enzyme activity in liver microsomes decreased in response to a fat-free diet. De novo enzyme synthesis was enhanced by dietary supplementation with linoleate but not palmitate. By contrast, De Schrijver and Privett (18) report that addition of safflower oil to a fat-free diet depressed Δ5 desaturase activity. This latter study appears to have induced a greater degree of essential fatty acid deficiency in its controls. Growth of human skin fibroblasts without serum lipids results in a progressive depletion of ω6 polyunsaturated fatty acids and the consequent synthesis of 20:3ω9 (13). Further studies with the cell culture system may help elucidate the apparently complex long term effects of dietary lipids on Δ5 desaturase activity.

The present study focuses on short term effects of exogenous fatty acids on Δ5 desaturation activity. All subsequent experiments thus used a 6-hr incubation period. In all cases, the fibroblast cells were previously grown for 4 days in lipid-free medium to enhance their desaturation of [^{14}C] eicosatrienoate.

Effects of Exogenous Fatty Acids

The effects of medium supplementation with free fatty acids on the metabolism of [^{14}C] eicosatrienoate are shown in Table 1. We find that total incorporation of the [^{14}C] 20:3 varies less than 15% with the addition of different fatty acids. Palmitate (16:0) does not significantly affect total Δ5 desaturation, although it appears to inhibit the elongation of [^{14}C] arachidonate to [^{14}C]22:4. Mild inhibition is seen with oleate (9c-18:1) and linoleate (9c, 12c-18:2), while inhibition with arachidonate (5c, 8c, 11c, 14c-20:4) is greater than 50%. Interestingly, the 2 *trans* fatty acids, elaidate (9t-18:1) and linoelaidate (9t,12t-18:2) are more inhibitory than their *cis* isomers. Elaidate is even more potent an inhibitor of Δ5 desaturation activity than is arachidonate, the product of the reaction.

Increased concentrations of free fatty acids in the culture medium have been shown to result in intracellular accumulation of triacylglycerol (15). In this study, we found that when the cells were incubated with either 40 μM elaidate or 40 μM oleate along with the 2.5 μM[^{14}C] eicosatrienoate, the percentage of ^{14}C-acyl groups esterified in triacylglycerol increased from 9.5% to 36% and 50% respectively. Both [^{14}C] 20:3 and the products of its desaturation were present in both phospholipids and triacylglycerol (data not shown). Consistent with previous findings in this laboratory (13), relatively more arachidonate and [^{14}C] 22:4 were esterified in phospholipids.

Figure 2 shows the effects of different concentrations of *trans* fatty acids on Δ5 desaturation. Inhibition by elaidate is seen at medium

TABLE 1

The Effects of Exogenous Fatty Acids on Incorporation and Desaturation of [^{14}C] Eicosatrienoate[a]

Fatty acid	[^{14}C] Incorporated[b] (n mol/flask)	^{14}C-Fatty acids 20:3	20:4	22:4	Desaturation[c] (%)
Control (no fatty acid)	5.22 ± 0.06	2.26	2.34	0.52	54.8
Palmitate (16:0)	5.12 ± 0.12	2.12	2.82	0.18	58.6
Oleate (9c-18:1)	5.68 ± 0.24	3.04	2.48	0.14	46.2
Linoleate (9c,12c-18:2)	4.90 ± 0.10	2.88	1.88	0.10	40.4
Arachidonate (5c,8c,11c,14c-20:4)	5.02 ± 0.14	3.52	1.14	0.30	28.7
Elaidate (9t-18:1)	5.36 ± 0.11	4.18	1.16	–	21.6
Linoelaidate (9t,12t-18:2)	5.28 ± 0.08	3.12	1.96	–	37.2

[a]Replicate flasks of GM-10 cells were established in lipid-free medium as in Figure 1. They were then preincubated with 3ml of medium containing delipidized serum and 40 μM fatty acid. After 2 hr, 1 ml of medium containing 0.5 μ Ci [1-^{14}C]eicosatrienoate was added to each flask. Final concentrations were 2.25 μM [^{14}C]eicosatrienoate and 30 μM non-radioactive fatty acid. The cells were harvested after a 6-hr incubation and their lipids analyzed as described in Materials and Methods.

[b]Values are means ± S.D. from analyses of 3 separate flasks from one of 2 similar experiments.

[c]Percentage desaturation calculated from Σ20:4 + 22:4/Total Incorporation.

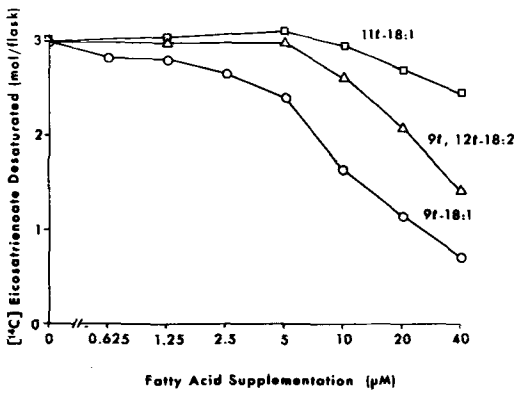

FIG. 2. The effect of concentration of exogenous *trans* fatty acids on the desaturation of [^{14}C]eicosatrienoate. As described in Table 1, replicate flasks were preincubated for 2 hr with *trans* fatty acids before addition of [^{14}C]20:3. ○ – ○, elaidate; □ – □, *trans* vaccenate, and △ – △, linoelaidate.

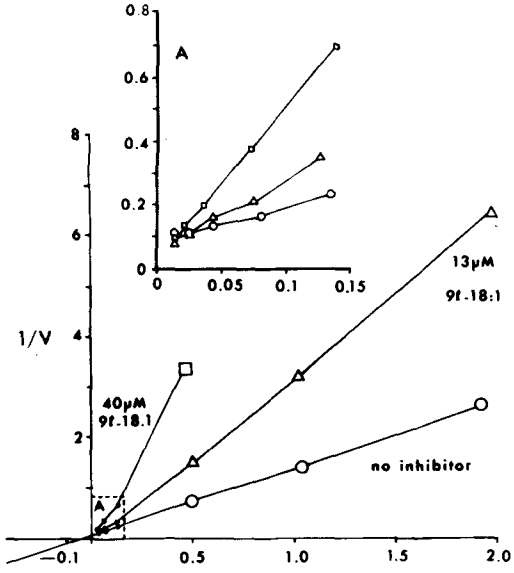

FIG. 3. The effects of varied substrate concentration on the desaturation of [^{14}C]eicosatrienoate in the presence and absence of elaidate. Replicate flasks were incubated for 6 hr with 0.2-30 μM [^{14}C]eicosatrienoate; concentrations above 4.5 μM 20:3 were obtained by addition of non-radioactive fatty acid. For transformation to the double reciprocal plot, the total incorporation of [^{14}C]20:3 nmol/flask was taken as substrate concentration, [S]. Velocity of the reaction was expressed as nmol 20:3 desaturated during the 6-hr incubation. The area with high substrate concentrations (1/[S] < 0.15), corresponding to 3.3-30 μM exogenous [^{14}C]eicosatrienoate, is magnified in the insert. ○ – ○, no elaidate; △ – △, 13 μM elaidate, and □ – □, 40 μM elaidate.

concentrations as low as 0.63 μM, one-fourth that of the [^{14}C]eicosatrienoate. Increased elaidate concentrations have a dose-dependent effect on Δ5 desaturation. By contrast, *trans* vaccenate (11t-18:1), a positional isomer of elaidate, has virtually no effect on the synthesis of arachidonate from [^{14}C]20:3. Although linoelaidate acts in an intermediate manner at 10-40 μM, lower concentrations are without measurable effect.

Our previous studies (12) have shown that *trans* vaccenate is readily incorporated into the glycerolipids of human skin fibroblasts. In addition, *trans* vaccenate acts similarly to elaidate in both its inhibition of de novo fatty acid synthesis and promotion of triacylglycerol accumulation. This seems to indicate that the markedly greater effects of elaidate on the desaturation of both [^{14}C]eicosatrienoate and [^{14}C]linoleate by human skin fibroblasts appear to be specific to the desaturation reactions. We also have performed similar experiments using human endothelial cells derived from umbilical vein (19). When these cells are incubated with [^{14}C]eicosatrienoate, the inhibition of Δ5 desaturation by elaidate is 2-3 fold that of linoelaidate; *trans* vaccenate has virtually no effect. Thus the relative effectiveness of the 3 *trans* fatty acids as inhibitors of Δ5 desaturation activity of human cells in culture is similar for 2 markedly different cell types.

There are 2 interesting differences in the effects of *trans* fatty acids on Δ6 and Δ5 desaturation activities of human skin fibroblasts. First, elaidate is a far more potent inhibitor of Δ5 desaturation than is linoelaidate, and its effects are seen at much lower inhibitor concentrations. The reverse relationship occurs for Δ6 desaturation. Second, the relative effectiveness of 9t-18:1 and 11t-18:1 on Δ5 desaturation in these cells is similar to that found in studies with rat liver microsomal preparations (10). The effects on Δ6 desaturation are quite different, with 11t-18:1 being more inhibitory than 9t-18:1 in microsomal studies and virtually without effect in the cell culture system.

Kinetics of Inhibition by Elaidate

We have examined the effects of elaidate on cells incubated with varied concentrations of [^{14}C]eicosatrienoate. In the absence of inhibitor, a double reciprocal plot of Δ5 desaturation activity vs. total incorporation gives a linear result (Fig. 3). Elaidate is inhibitory in a dose-dependent manner over a wide range of eicosatrienoate concentrations. As seen in Figure 3, the inhibitory effect of elaidate can be overcome at high substrate concentrations.

Mahfouz et al. (10) have pointed out that since both substrate and products are incorporated into a variety of glycerolipids, a Lineweaver-Burk plot should not strictly apply for the microsomal fatty acyl desaturase reactions. They found, however, that the microsomal system did give linear double reciprocal plots for fatty acyl Δ5 desaturation indicating that the desaturation step is rate limiting. In their assays *trans* octadecenoates acted as classic competitive inhibitors. Use of exogenous fatty acids by intact cells is clearly more complex than a microsomal assay, and the incubation times are longer. The convergent lines obtained with intact cells (Fig. 3) are suggestive of competitive inhibition. However, examination of the data for high substrate concentrations (Fig. 3A) indicates that the lines do indeed converge at a finite substrate concentration and actually are curves at low 1/[s] values. This may be due to the increased importance of other factors such as relative scarcity of CoA or accumulation of neutral lipid. Alternatively, our assay may actually measure 2 reactions, both the conventional eicosatrienoyl CoA desaturase and the eicosatrienoyl lecithin desaturase described by Pugh and Kates (20).

Influence of Position of the *Trans* Double Bond

The different results obtained with elaidate and *trans* vaccenate (Fig.2) would seem to indicate that the position of the *trans* double bond is important in determining the effects of the fatty acid on Δ5 desaturation. To investigate this further we examined the effects of a series of commercially available *trans* monounsaturated fatty acids. As seen in Table 2, there is no correlation between the position of the *trans* double bond as counted from the carboxyl carbon (9t, 10t, or 11t) and effectiveness as an inhibitor. Thus palmitoleate (9t-16:1) does not inhibit the desaturation of [^{14}C] eicosatrienoate, while elaidate (9t-18:1) does. When the *trans* fatty acids are grouped by the position of the *trans* double bond from the methyl carbon, a definite pattern is seen. All the ω9 *trans* fatty acids tested are potent inhibitors of Δ5 desaturation activity in human skin fibroblasts; the ω7 *trans* fatty acids are not particularly inhibitory.

This selectivity in the inhibitory effects of *trans* monoenoic fatty acids is quite puzzling. Substrate specificity of mammalian desaturases appears to involve recognition of the carboxyl end of the molecule (21). The major substrate requirements of the Δ5 desaturase involve 11c and 14c double bonds, while the 8c bond enhances reactivity. Pollard et al. (22) have shown very low levels of Δ5 desaturation of 9t-20:1 but not 11t-18:1 by rat liver microsomes. They found, however, that desaturation of 9t-20:1 was 7-fold greater than 9t-18:1 Our data (Table 2) indicates that 9t-20:1 is not as effective an inhibitor as is 9t-18:1. Thus, there is no correlation between the extent of Δ5 desaturation of a *trans* monoenoate in the microsomal assays

TABLE 2

The Effects of Different *trans* Monoenoic Fatty Acids on the Incorporation and Desaturation of [^{14}C] Eicosatrienoate[a]

Fatty acid	Concentration	Incorporation of [^{14}C] 20:3 (n mol/flask)	Desaturation (%)
Control (no fatty acid)	–	5.62 ± 0.26	39.2
9t-16:1 (ω7)	13 μ M	4.68 ± 0.10	46.2
10t-17:1 (ω7)	13 μ M	5.40 ± 0.14	41.8
11t-18:1 (ω7)	13 μ M	6.00 ± 0.08	39.0
9t-18:1 (ω9)	13 μ M	5.66 ± 0.08	24.8
10t-18:1 (ω9)	13 μ M	6.12 ± 0.02	31.3
11t-20:1 (ω9)	13 μ M	6.32 ± 0.03	34.6
9t-16:1 (ω7)	40 μ M	5.42 ± 0.16	43.7
10t-17:1 (ω7)	40 μ M	5.94 ± 0.11	34.7
11t-18:1 (ω7)	40 μ M	6.54 ± 0.08	29.4
9t-18:1 (ω9)	40 μ M	6.62 ± 0.13	14.7
10t-19:1 (ω9)	40 μ M	6.50 ± 0.20	13.7
11t-20:1 (ω9)	40 μ M	6.86 ± 0.22	19.5

[a]Replicate flasks of GM-10 cells were preincubated with *trans* fatty acids for 2 hr as described in Table 1. 0.5 μ CI [1-^{14}C] Eicosatrienoate/flask was then added for the 6-hr incubation. Final *trans* fatty acid concentrations are indicated above; other procedures as in Table 1.

and its inhibition of eicosatrienoate desaturation activity in intact cells. If these *trans* monosaturates do indeed act as competitive inhibitors, (10), elucidation of their specificity may contribute to our understanding of the structural configuration of the active site.

A decrease in liver microsomal Δ5 desaturase activity has been observed in rats fed partially hydrogenated marine oils but not partially hydrogenated peanut oil (21,22). Svensson (23) has suggested that this effect may be due to *trans* eicosenoic and docosenoic fatty acids in the marine oil products. In our system 11t-20:1 is only slightly less effective than elaidate as an inhibitor of the desaturation of [^{14}C]eicosatrienoate. 11t-Eicosatrienoate is, however, significantly less effective than elaidate as an inhibitor of the desaturation of [^{14}C]linoleate (Rosenthal, M.D.; Doloresco, M.A., and Banerjee, N., unpublished observations). It would be of interest to examine the effects of other isomeric *trans* eicosenoates. A *trans* fatty acid or fatty acid analogue which is still more selective in its effects on Δ5 desaturation might have potentially useful effects on the metabolism of eicosatrienoate and arachidonate in vivo.

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REFERENCES

1. Brenner, R.R. (1974) Mol. Cell. Biochem. 3:41-52.
2. Moncada, S., and Vane, J.R. (1979) N. Engl. J. Med. 300:1142-1147.
3. Crawford, M.A., Casperd, N.M., and Sinclair, A.J. (1976) Comp. Biochem. Physiol. 54B:395-401.
4. Hassam, A.G., and Crawford, M.A. (1978) Lipids 13:801-803.
5. Stone, K.J., Willis, A.L., Hart, M,, Kirtland, S.J., Kernoff, P.B.A., and McNicol, G.P. (1979) Lipids 14:174-180.
6. Kinsella, J.E., Bruckner, G., Mai, J., and Shimp, J. (1981) Am. J. Clin. Nutr. 34:2307-2318.
7. Hill, E.G., Johnson, S.B., Lawson, L.D., Mahfouz, M.M., and Holman, R.T. (1982) Proc. Natl. Acad. Sci. 79:953-957.
8. Kurata, N., and Privett, O.S. (1980) Lipids 15:1029-1036.
9. Bruckner, G., Shimp, J., Goswami, S., Mai, J., and Kinsella, J.E. (1982) J. Nutr. 112:126-135.
10. Mahfouz, M.M., Johnson, S., and Holman, R.T. (1980) Lipids 15:100-107.
11. Mahfouz, M.M., Johnson, S., and Holman, R.T. (1981) Biochim. Biophys. Acta. 663:58-68.
12. Rosenthal, M.D., and Whitehurst, M.C. (1983) Biochim. Biophys. Acta. 753:450-459.
13. Rosenthal, M.D., and Whitehurst, M.C. (1982) J. Cell. Physiol. 113:298-306.
14. Rothblat, G.H., Arbogast, L.Y., Ouellette, L., and Howard, B.V. (1976) In Vitro 12:554-557.
15. Rosenthal, M.D. (1981) Lipids 16:173-182.
16. Slayback, J.R.B., Cheung, L.W.Y., and Geyer, R.P. (1977) Anal. Biochem. 83:372-384.
17. de Gomez Dumm, I.N.T., De Alaniz, M.J.T., and Brenner, R.R. (1983) Lipids 18:781-788.
18. De Schrijver, R., and Privett, O.S. (1983) J. Nutr. 113:2217-2222.
19. Rosenthal, M.D., and Whitehurst, M.C. (1983) Biochim. Biophys. Acta. 750:490-496.
20. Pugh, E.L., and Kates, M. (1979) Lipids 14: 159-165.
21. James, A.T. (1977) Adv. Exp. Med. Biol. 83: 51-74.
22. Pollard, M.R., Gunstone, F.D., James, A.T., and Morris, L.J. (1980) Lipids 15:306-314.
23. Svensson, L. (1983) Lipids 18:171-177.

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Intestinal Metabolism of Plasma Free Fatty Acids in Streptozotocin Diabetic Rats

F. RENNER[a], G. SCHERNTHANER[b] and A. GANGL*,[a], [a]Department of Gastroenterology and Hepatology and [b]Department of Medicine, University of Vienna, Allgemeines Krankenhaus, A-1090 Vienna, Austria

ABSTRACT

Moderate insulin deficiency was reported to be accompanied by an increased production of intestinal very low density lipoprotein (VLDL) triglyceride in the rat. Because plasma free fatty acids (FFA) are incorporated into triglyceride by intestinal mucosa of rats and humans and plasma FFA are increased in insulin-deficient diabetes mellitus, we investigated several aspects of the intestinal metabolism of plasma FFA in diabetic rats. All experiments were performed on the third day following the i.v. injection of streptozotocin (45 mg/kg body weight) or buffer alone. A (^{14}C)palmitic acid-rat serum complex was rapidly injected intravenously and its initial uptake by small bowel mucosa, the intracellular incorporation into lipids and water soluble metabolites and the specific radioactivity of triglycerides of mucosal homogenates was determined. No significant differences could be found between diabetic and control rats at 2 and 5 min after ^{14}C-palmitate i.v., suggesting that neither the influx of plasma free fatty acids into intestinal mucosal cells nor their initial intracellular metabolic pathways are significantly altered in moderately diabetic rats. A pronounced decrease in intestinal mucosal triglyceride at 10 min after ^{14}C-palmitate i.v. might be interpreted as indirect evidence for an enhanced triglyceride efflux from intestinal mucosa into mesenteric lymph in diabetic rats.
Lipids 19:875-879, 1984.

INTRODUCTION

Recent studies have revealed increasing evidence that insulin deficiency states are accompanied by major changes in intestinal lipid metabolism. Pancreatomized dogs maintained on a fat free diet developed hypertriglyceridemia associated with the appearance of chylomicron-like particles in the mesenteric lymph (1). More recently, an abnormal accumulation of lipids was described micromorphologically in intestinal mucosa of children with poorly controlled insulin deficient diabetes mellitus (2). Finally, a contribution of intestinal mucosa to the overall production of VLDL triglycerides in rats with moderate insulin deficiency was demonstrated (3,4). Since plasma free fatty acids (FFA) also are esterified to endogenous triglycerides by intestinal mucosa (5,6) and plasma FFA are increased in insulin deficiency states (7), it was speculated that in this special condition plasma FFA might be an important source of intestinal triglyceride production (3).

This study was undertaken to follow the intestinal metabolic fate of plasma FFA in diabetic rats.

MATERIALS AND METHODS

Animals and Their Diabetogenic Treatment

Unanaesthetized male albino rats (body weight 280-350 g per rat) were used in all experiments. During the experimental period animals were fed standard rat chow ad libitum combined with free access to water until 12 hrs before blood was drawn (day 1 and 2) or rats were decapitated (day 3). Streptozotocin (Serva Feinbiochemica GmbH., Heidelberg, West Germany) was freshly dissolved in citrate buffer pH 4.5 at 4 C before each experiment and then 1 ml of this solution, or buffer alone (controls), was injected into the tail vein of the conscious rat. The dose of streptozotocin was 45 mg/kg body weight. After 24 and 48 hrs blood for monitoring glucose, triglyceride and FFA concentrations was gained by scarification of the tail tip.

Measurement of plasma immunoreactive insulin levels was performed in the fasting state before and after streptozotocin administration.

Preparation of (^{14}C)palmitic Acid-rat Serum Complex

About 20 μCi (U-^{14}C)palmitic acid (735 mCi/mmol, New England Nuclear, Boston, Massachusetts; radiochemically > 98% pure) dissolved in 20 μl of methylethyl ketone, was neutralized with a slight excess of ethanolic KOH, slowly mixed with 3 ml rat serum, and diluted 1:1 with 0.85% saline. This preparation was made immediately before each experiment, and about 1.0 ml of this preparation (0.5 ml serum) was injected per rat. The successful use of such a (^{14}C) palmitic acid-rat serum complex has been described earlier (5).

Experimental Procedure and Tissue Preparation

The (^{14}C)palmitic acid-rat serum complex

*To whom correspondence should be addressed.

was injected acutely into a tail vein of conscious rats. After decapitation at specified time intervals (2, 5 and 10 min after injection), blood was collected and then the abdomen was opened immediately. The small intestine from duodenum to coecum was removed and flushed with 50 ml of ice-cold saline. The time from decapitation to completion of saline flush never exceeded 90 sec. The isolated small intestine was divided into proximal (jejunal) and distal (ileal) halves, the whole mucosa from the former (jejunal) segment extruded on a chilled glass plate and homogenized in a Teflon-glass homogenizer after adding 3 vol of methanol to interrupt any lipolytic activity.

ANALYTICAL METHODS

Lipids were extracted from mucosal homogenates and whole serum by the method of Folch et al. (8). Lipid classes were separated on precoated TLC plates (Silica gel, 0.25 mm EM reagents, E. Merck, Darmstadt, West Germany) and eluated after identification by comparison with standards as described elsewhere (5). These eluates were used for determination of fatty acids (8) and triglycerides (10). The protein content of the mucosal homogenates was determined by the method of Lowry et al. (11). Blood glucose was measured by an enzymatic method (12).

Serum levels of insulin were determined by radioimmunoassay as described by Heding (13), using ethanol for the precipitation of the antigen-antibody complex.

Radioassays

Aliquots of homogenates (0.2 ml) were assayed for radioactivity in liquifluor toluene solution (New England Nuclear) containing 10% Biosolv (Beckman Instruments, Inc., Fullerton, California) in a Beckman liquid scintillation system (model LS 315oT). For lipid soluble extracts, Biosolv was not added. Water soluble radioactivity in intestinal mucosa was calculated by subtracting the lipid soluble radioactivity from the radioactivity in whole mucosal homogenates. Quenching was corrected for by an automatic external standard, and results were expressed as disintegrations per minute.

Calculation of Data

To permit comparison of results among rats injected with slightly different amounts of isotope, all radioactivity data were normalized to an injection of 1.0 μCi. All results are expressed as mean ± 1 standard error. The two groups (streptozotocin treated vs. controls) were compared by Student's t-test.

RESULTS

Serum levels of glucose, triglycerides and FFA during the experimental period are shown in Figure 1. Blood glucose was significantly increased in the streptozotocin treated rats as compared with controls at all time points investigated. Also, triglycerides and FFA were elevated in the diabetic rats, almost always reaching statistical significance. Fasting serum insulin levels were 0.68 ± 0.07 ng/ml in the streptozotocin treated rats and 0.75 ± 0.15 ng/ml in controls, the slight difference being insignificant. To evaluate whether for studying these moderately diabetic rats 3 days after initiation of diabetes steady state conditions have been reached already, in additional experiments serum glucose, free fatty acids and triglycerides were measured 4 (n = 12) and 7 - 9 days (n = 14) after streptozotocin. Glucose was elevated constantly on days 3 and 4 (256 ± 34 mg/dl and 240 ± 34 mg/dl; mean ± SEM) and declined somewhat on days 7 - 9 (161 ± 14 mg/dl). Serum free fatty acids were almost identical on days 3 and 4 (0.69 ± 0.05 mmol/l and 0.65 ± 0.05) and showed a moderate decline on days 7 - 9 (0.58 ± 0.04 mmol/l). Triglycerides were not significantly different on day 3 (0.98 ± 0.25 mmol/l), day 4 (0.66 ± 0.12) and days 7 - 9 (0.71 ± 0.22).

Intestinal mucosal triglyceride and fatty acid concentrations as determined on day 3 after i.v. injection of streptozotocin diabetic and control rats are shown in Table 1.

Uptake and Metabolism of Plasma FFA by Intestinal Mucosa (Table 2)

Since it was shown in earlier studies (5) that no major differences exist in mucosal metab-

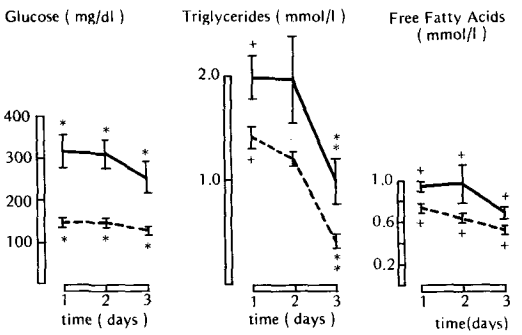

FIG. 1. Serum levels of glucose, triglycerides and plasma free fatty acids in rats 1, 2 and 3 days after i.v. injection of streptozotocin (45 mg/kg body weight, n = 18, —) and in controls (---). Blood was drawn after an overnight fast on day 1 and 2 from the tail tip and on day 3 after decapitation. M ± SEM. *$p < 0.005$, **$p < 0.02$, +$p < 0.05$ (t-test).

TABLE 1

Intestinal Mucosal Triglycerides and Free Fatty Acids of Diabetic and Control Rats (μ mole/g mucosal protein)

	Diabetics	Controls
Triglycerides	16.8 ± 2.9	13.5 ± 1.8
Free fatty acids	6.8 ± 0.7	7.2 ± 0.9

After an overnight fast diabetic (n = 18) and control rats (n = 17) were decapitated on day 3. Intestinal mucosal homogenates were assayed for triglycerides and free fatty acids. See also methods. M ± SEM. Differences between diabetics and controls are statistically not significant (t-test).

olism of plasma FFA between proximal and distal small intestine, only the proximal portion was investigated. Intestinal mucosal radioactivity 2 min after intravenous injection of (^{14}C) palmitic acid was 6156 ± 350 dpm/g wet weight in diabetic rats and 6560 ± 487 dpm/g wet weight in control rats. Apparently there was no major difference. Since plasma free fatty acid specific activities at this early time also were not significantly different in diabetic and control rats (8517 ± 1165 dpm × μmol^{-1} vs 11742 ± 1483, mean ± SEM), one can assume that the initial mucosal uptake is not altered in diabetic rats.

At 5 and 10 min a moderate but steady increase in mucosal radioactivity was observed. However, no significant difference existed between diabetic and control rats at the specified intervals.

The distribution of (^{14}C) among intestinal mucosal metabolites also is shown in Table 2. The major part of radioactivity was incorporated into water soluble metabolites (about 1/3) and phospholipids (about 1/3); to a minor extent (^{14}C) was incorporated into endogenous triglycerides (about 1/5). This general pattern was rather constant in diabetic and control rats at 2 and 5 min after i.v. injection of the (^{14}C) palmitate-rat serum complex. Only at 10 min significantly more ^{14}C was found in water soluble metabolites and less radioactivity in mucosal triglycerides of diabetic rats. This observation is discussed below.

To rule out the possibility that (^{14}C) labeled triglycerides isolated from the small bowel mucosa do not reflect de novo synthesis by intestinal epithelial cells but represent only a contamination by hepatogenous serum triglycerides, the specific activities of triglycerides in mucosa and serum were calculated. As shown in Figure 2 the specific activities of serum triglycerides were significantly less than those of mucosal triglycerides at 2, 5 and 10 min, thus excluding any significant contribution by serum (^{14}C) triglycerides to intestinal mucosa (^{14}C) triglycerides and confirming our earlier findings in rat (5) and man (6) that plasma FFA are incorporated into triglycerides by intestinal mucosa. No significant difference in this intestinal pathway existed between diabetic and control rats.

DISCUSSION

Studying moderately diabetic rats 3 days after streptozotocin injection seems appropriate since adaptation changes—a well known phenomenon when relatively small doses of streptozotocin are used (14)—have not occurred yet but plasma free fatty acids already exhibited steady state conditions.

The finding of a similar intestinal lipid content in diabetic and control rats is in good agreement with earlier findings in man, where intestinal mucosa also contained similar amounts of lipids both in well controlled juvenile or maturity onset diabetes and in normal subjects (15).

The initial uptake of (^{14}C) into intestinal mucosa after i.v. injection of (^{14}C) palmitate is not different between diabetic and normal rats, indicating that plasma FFA influx into the intracellular space is not altered in moderately insulin deficient rats. Also, the intracellular esterification and oxidation pathways of these plasma derived FFA cannot be significantly abnormal in diabetic rats, because the pattern of lipid radioactivity at 2 and 5 min was quite similar in both groups investigated and consistent with earlier data in normal rats (5). The finding that significantly less mucosal triglyceride radioactivity was detected 10 min after (^{14}C) application in diabetic rats is difficult to interpret at the moment. Because a small, but significantly higher amount of (^{14}C) was recovered in mucosal water soluble metabolites of diabetic rats at the same time, this could mean that plasma derived (^{14}C) palmitate was oxidized to a higher extent within the preceding 10 min at the expense of incorporation into mucosal triglycerides.

It is unlikely, however, that this effect would show only relatively late at 10 min but not at 2 and 5 min after the i.v. injection of (^{14}C) palmitic acid. Therefore, an alternate explanation seems more attractive: The recent description of a significantly higher contribution of endogenous fatty acids to the enhanced production of intestinal VLDL triglyceride fatty acids in diabetic rats during fasting (4), and the fact that we could not find a significant difference in the intestinal mucosal triglyceride concentrations of diabetic and control rats (Table 1) can be reconciled by the assumption of an increased intestinal secretion of triglyc-

TABLE 2

Initial Uptake and Metabolism of i.v. (^{14}C)Palmitic Acid by Intestinal Mucosa of Diabetic and Control Rats

	2 min		5 min		10 min	
	Diabetes n = 8	Control n = 7	Diabetes n = 5	Control n = 5	Diabetes n = 5	Control n = 5
Mucosal Radioactivity (dpm/g mucosa)	6156 ± 350	6560 ± 487	7959 ± 371	7232 ± 518	9800 ± 394	8096 ± 749
H_2O soluble metabolites % of mucosal radioactivity	31.8 ± 1.2	33.1 ± 1.5	38.4 ± 1.9	37.7 ± 1.2	36.7 ± 2.1*	30.5 ± 0.9*
Lipids (total) % of mucosal radioactivity	68.2 ± 1.2	66.7 ± 1.4	61.6 ± 1.9	62.3 ± 1.2	63.3 ± 2.1*	69.5 ± 0.9*
Phospholipids	36.4 ± 1.0	34.5 ± 1.8	30.9 ± 1.2	36.4 ± 3.2	40.9 ± 2.1	38.3 ± 1.3
Triglycerides	22.9 ± 2.1	23.8 ± 1.6	25.1 ± 2.1	20.3 ± 1.7	18.3 ± 1.7†	26.8 ± 0.9†
Cholesterolester	2.6 ± 0.4	1.7 ± 0.2	1.3 ± 0.1	1.3 ± 0.1	1.3 ± 0.2	1.1 ± 0.2
Free Fatty Acids	2.2 ± 0.2	2.8 ± 0.4	1.8 ± 0.4	1.4 ± 0.1	1.1 ± 0.1	1.3 ± 0.2
Diglycerides	4.1 ± 0.5	3.9 ± 0.5	2.6 ± 0.4	1.9 ± 0.1	1.6 ± 0.2	1.9 ± 0.5

(^{14}C)palmitic acid was rapidly injected intravenously in diabetic and control rats after an overnight fast. At 2, 5 and 10 min rats were decapitated; intestinal mucosa was prepared and analyzed for (^{14}C) in whole homogenates and individual lipid classes and estimated in the water soluble fraction (see methods). Mean ± SEM; *$p < 0.05$, †$p < 0.005$ (t-test).

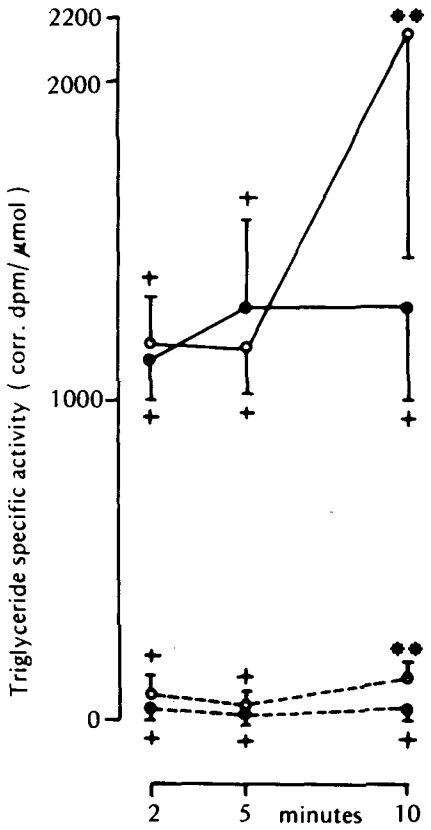

FIG. 2. Specific activity (dpm/μmole) of triglycerides in intestinal mucosa (—) and serum (---) of diabetic (•) and control (○) rats after i.v. (^{14}C)palmitic acid. After an overnight fast rats received (^{14}C) palmitate rapidly intravenously, and specific activity of triglycerices in mucosa and serum was determined after decapitation at 2 min (8 diabetic, 7 control rats), 5 min (5 diabetic, 5 control rats) and 10 min (5 diabetic, 5 control rats). Mean ± SEM. Statistically significant differences between specific activities in serum and mucosa are designated with $^{+}p < 0.02$ and $^{**}p < 0.005$ (t-test). The differences between diabetic and control rats in serum and mucosal specific activities were not statistically significant.

eride rich lipoproteins in diabetic rats. Thus, our finding of significantly less triglyceride radioactivity at 10 min after the application of (^{14}C) palmitic acid in diabetic rats could be interpreted as indirect evidence of an accelerated efflux of labeled triglyceride from intestinal epithelial cells into mesenteric lymph. This speculation could be confirmed only by direct measurement of lymph triglyceride radioactivity, which was not performed. Nevertheless, there is no evidence that plasma free palmitic acids play a major role in the enhanced intestinal VLDL triglyceride production in diabetic rats with moderate insulin deficiency. One cannot rule out the possibility that the intestinal metabolism of plasma FFA is altered when diabetic animals are completely insulin deficient.

REFERENCES

1. Steiner, G.S., Poapst, M, and Davidson, J.K. (1975) Diabetes 24, 263-71.
2. Bobo, R.C., Partin, J.C., Schubert, W.K., and Saalfeld, K. (1977) Am. J. Dis. Child 131, 962-9.
3. Risser, T.R., Reaven, G.M., and Reaven, E.P. (1978) Diabetès 27, 902-8.
4. Popper, D.A., and Shiau, Y.F. (1983) Gastroenterology 84, 1275A.
5. Gangl, A., and Ockner, R.K. (1975) J. Clin. Invest. 55, 803-13.
6. Gangl, A., and Renner, F. (1978) Gastroenterology 74, 847-50.
7. Scow, R.O., and Chernick, S.S. (1970) In Comprehensive Biochemistry, Florkin, M., and Stotz, E.H., eds., Amsterdam, London, New York: Elsevier Publishing Company, pp. 19-49.
8. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497-509.
9. Regouw, B.J.M., Cornelissen, P.J.H.C., Helder, R.A.P., et al. (1971) Clin. Chim. Acta 31, 187-95.
10. Eggstein, M., and Kreutz, F.H. (1966) Klin. Wochenschr. 44, 262-7.
11. Lowry, O., Rosebrough, N.J., Farr, A.L., et al. (1951) J. Biol. Chem. 193, 265-75.
12. Slein, M.W. (1963) In Methods of Enzymatic Analysis, H.V. Bergmeyer, ed. Academic, New York, p. 117.
13. Heding, L.G. (1972) Diabetologia 8, 260-6.
14. Junod, A., Lambert, A.E., Stauffacher, W., and Renold, A.E. (1969) J. Clin. Invest. 48, 2129-2139.
15. Renner, F., Pavelka, M., Schernthaner, G., and Gangl, A. (1981) Klin. Wochenschr 59, 561-5.

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METHODS

Quantitative Analysis of Triglyceride Species of Vegetable Oils by High Performance Liquid Chromatography via a Flame Ionization Detector

F.C. PHILLIPS[a], W.L. ERDAHL[a], J.A. SCHMIT[b], and O.S. PRIVETT[a]*, [a]*The Hormel Institute, University of Minnesota, Austin MN 55912, and* [b]*E.I. Dupont de Nemours and Co., Wilmington DE 19898*

ABSTRACT

A method for the quantitative analysis of triglyceride species composition of vegetable oils by reversed-phase high performance liquid chromatography (RP-HPLC) via a flame ionization detector (FID) is described. Triglycerides are separated into molecular species via Zorbax chemically bonded octadecylsilane (ODS) columns using gradient elution with methylene chloride in acetonitrile. Identification of species is made by matching the retention times of the peaks in the chromatogram with the order of elution of all of the species that could be present in the sample on the basis of a random distribution of the fatty acids and comparision of experimental and calculated theoretical carbon numbers (TCN). Quantitative analysis is based on a direct proportionality of peak areas. Differences in the response of individual species were small and did not dictate the use of response factors. The method is applied to cocoa butter before and after randomization, soybean oil and pure olive oil.
Lipids 19:880-887, 1984.

INTRODUCTION

Considerable progress has been made in recent years on the separation of triglyceride species by reversed-phase high performance liquid chromatography (RP-HPLC) (1-9). Quantitative analysis generally is performed on isolated fractions by gas liquid chromatography (GLC) of methyl esters using an internal standard as described by Plattner (2) and Wada (10) and as used in glyceride analysis in conjunction with lipase hydrolysis and thin layer chromatography (TLC) (11-13). Quantitative analysis directly by ultraviolet (UV) (14) or refractive index (RI) (15) detectors usually is not made because of the need for response factors for all of the individual species. These detectors also place limitations on the solvents that can be used in the chromatography.

In a recent report (16) we described the separation by RP-HPLC of triglyceride species differing by one double bond or one methylene group, as well as of 2 and 4 component critical pair mixtures. In the present study the quantitative analysis of the molecular species of triglycerides based on a direct proportionality of peak areas using a flame ionization detector (FID) developed in this laboratory (17) is demonstrated on standard mixtures of pure triglycerides and applied to several vegetable oils.

*To whom correspondence should be addressed.

MATERIALS AND METHODS

Triglycerides. The following highly purified simple triglycerides were obtained from Nu-Check Prep, Inc., Elysian, Minnesota: trilaurin (36:0), trimyristin (42:0), tripalmitin (48:0), tripalmitolein (48:3), tristearin (54:0), triolein (54:3), trilinolein (54:6) and trilinolenin (54:9).

Triglyceride reference mixtures. Four mixtures, A, B, C and D, were prepared for identification purposes by randomization of equal amounts of the following pure triglycerides: A, tripalmitin, trilinolein and trilinolenin; B, tripalmitin, triolein and trilinolein; C, tripalmitin, triolein and tristearin, and D, triolein, trilinolein and trilinolenin.

The randomizations were carried out by heating equal amounts of the pure triglyceride mixtures for 4 hr at 100 C under an atmosphere of nitrogen with a sodium methoxide catalyst as previously described (18). The reaction mixtures were purified by gravity flow silicic acid column chromatography in which the nonpolar material was eluted with low boiling petroleum ether, following which the triglycerides were collected as a single fraction by elution with methylene chloride. Each of the above mixtures contained 10 molecular species as follows: Mixture A. P_3, L_3, Ln_3, P_2L_1, P_1L_2, P_2Ln_1, P_1Ln_2, L_2Ln_1, L_1Ln_2, $P_1L_1Ln_1$; P = palmitate, L = linoleate, and Ln = linolenate; Mixture B. P_3, O_3, L_3, P_2O_1, P_1O_2, P_2L_1, P_1L_2,

O_2L_1, O_1L_2, $P_1O_1L_1$; P = palmitate, O = oleate, and L = linoleate; Mixture C. P_3, O_3, S_3, P_2O_1, P_1O_2, P_2S_1, P_1S_2, O_2S_1, O_1S_2, $P_1O_1S_1$; P = palmitate, O = oleate, and S = stearate; Mixture D. O_3, L_3, Ln_3, O_2L_1, O_1L_2, O_2Ln_1, O_1Ln_2, L_2Ln_1, L_1Ln_2, $O_1L_1Ln_1$; O = oleate, L = linoleate, and Ln = linolenate.

Cocoa butter and soybean oil were commercial samples. Randomized cocoa butter was prepared from the natural sample as described above. The triglycerides of these oils also were purified by gravity flow column chromatography with silicic acid. Samples of pure olive oil were obtained from Dr. Eduardo Vioque of the Institute de La Grassa sus Derivados (C.S.I.C.), Seville, Spain, and Professor Enzo Fedeli, Centro Nazionale Per La Lipochimica, Milano, Italy, and used as received.

Fatty acid composition of the oils was determined on methyl esters, prepared as described by Christie (19), using a Hewlett Packard Model 5840A gas chromatography with a 12' x 0.125" o.d. column of 10% Silar 10 C on 100-200 mesh Gas Chrom Q (Applied Science, State College, Pennsylvania). The column temperature was programmed from 200-225 C at 2.0 C per min with a flow rate of helium of 10 ml/min. The fatty acid analyses of the oils studied here are shown in Table 1.

Lipase hydrolysis. The method used in this work is described by Privett and Nutter (11). It is a refinement of that described by Mattson and Volpenheim (20) which has been used extensively in methods for the determination of triglyceride structure.

RP-HPLC. This technique was carried out with a Spectra Physics Model 3500 B liquid chromatograph equipped with two 4.6 mm x 25 cm Zorbax columns of chemically bonded ODS connected together and a FID as previously described (16). The columns were obtained from E.I. Dupont de Nemours and Co., Wilmington, Delaware. Peak areas were recorded automatically and per cent composition determined with an IMSAI 8080 microprocessor-integrator system. The chromatography was carried out with a linear gradient elution program starting with various concentrations of methylene chloride but generally 30% by vol in acetonitrile. The concentration of methylene chloride was increased until all of the components were eluted. In order to demonstrate the application of the FID in conjunction with large bore columns, several experiments were carried out with a Golden Series Zorbax ODS column, packed with 3 μm diameter porous spherical microspheres in a 6.2 mm x 8.0 cm configuration, also obtained from E. I. Dupont de Nemours and Co., Wilmington, Delaware.

Solvents. Methylene chloride was a reagent grade purchased from local suppliers and purified by a preliminary distillation followed by shaking in a separatory funnel with concentrated sulfuric acid several times, dilute sodium carbonate and finally with water. The washed solvent was dried over calcium chloride and redistilled in an all-glass still.

Acetonitrile was a reagent grade obtained from Fischer Scientific Co., Fairlawn, New Jersey. It was treated with phosphorus pentoxide and fractionally distilled through a 2-meter Hyper-cal Podbielniak column at a reflux ratio of 20:1. This procedure removed nonvolatile contaminants and insured uniformity of the solvent from batch to batch.

Flame ionization detector (FID). The detector is described in previous work (17). Briefly, the eluent from the column is applied to the moving transport device which is a perforated belt whose construction features have been described previously (17). The solvent is removed in the evaporator which is maintained at 200 C in a stream of nitrogen and collected in a dry ice trap. The sample remains as a residue on the belt and is transported into the reactor, which is heated to 600 C, where it is converted to vol-

TABLE 1

Fatty Acid Composition of Natural Oils

Fatty acid	Cocoa butter (%)	Soybean oil (%)	Italian olive oil (%)	Spanish olive oil (%)
16:0[a]	24.94 ± 0.36[b]	10.40 ± 0.03	12.08 ± 0.03	13.26 ± 0.24
16:1	0.53 ± 0.02	0.24 ± 0.01	1.02 ± 0.02	1.37 ± 0.02
18:0	34.52 ± 0.22	4.19 ± 0.02	2.57 ± 0.01	3.41 ± 0.03
18:1	35.36 ± 0.22	22.49 ± 0.03	74.12 ± 0.10	72.36 ± 0.18
18:2	4.11 ± 0.04	53.96 ± 0.04	8.64 ± 0.02	8.28 ± 0.05
18:3	0.17 ± 0.01	7.12 ± 0.01	0.71 ± 0.02	0.63 ± 0.04
20:0	–	0.57 ± 0.01	0.40 ± 0.02	0.30 ± 0.03

[a]Number before colon = number of carbon atoms; number after colon = number of double bonds.

[b]Mean ± S.D.

atile hydrocarbons by reaction with hydrogen. The products of the reaction are swept into the stack in a mixture of nitrogen and hydrogen and combusted in a flame of hydrogen at the outlet. The current that is produced upon ionization of the sample in the flame is amplified by a Keithley (Model 417K) electrometer. The signal is recorded on a strip chart recorder and passed into an integrator which stores it until it is processed by the IMSAI microcomputer and printed out. The raw data is converted to per cent composition on the basis of a proportionality of peak areas by means of a BASIC software program.

RESULTS

Reference mixtures A, B and C were used

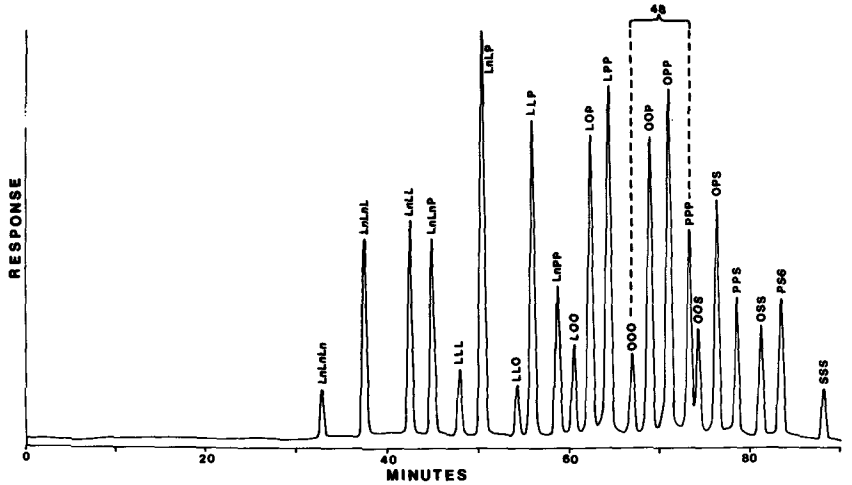

FIG. 1. Separation of a mixture of randomized samples A, B and C; column - two 250 X 4.6 mm, 5 μm, Zorbax C_{18} ODS columns coupled in series; mobile phase - 120 min linear gradient from 30 to 60% methylene chloride in acetonitrile; flow rate - 0.8 ml per min; detector - flame ionization. Species identification as indicated with Ln = linolenate; L = linoleate; O = oleate; P = palmitate; S = stearate (the order of designation does not indicate positional isomers).

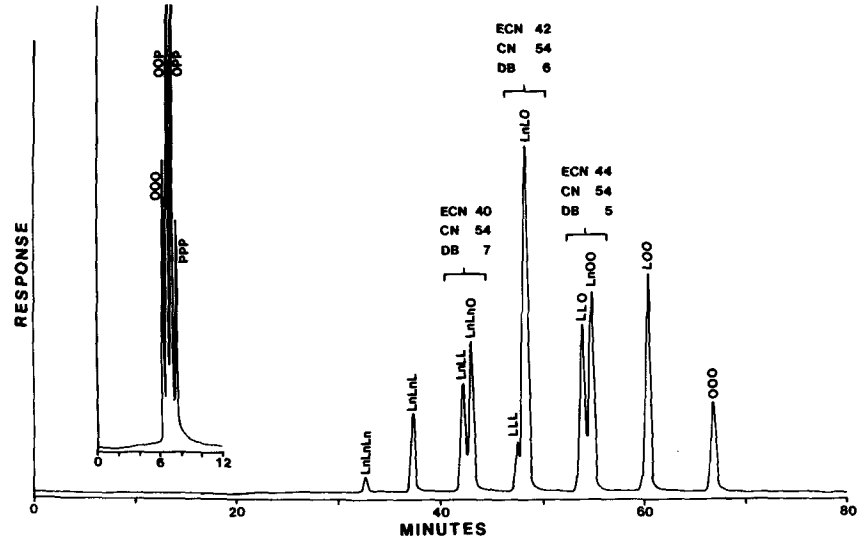

FIG. 2. Separation of randomized mixture D; conditions and species designations as in Fig. 1. Insert is the separation of a 4-component critical pair mixture using an isocratic solvent system of 40% methylene chloride in acetonitrile and a column 80 mm X 6.2 mm, 3 μm, Zorbax ODS at 1.2 ml per min with an effluent split of 1:1.

separately and in combination. There are 10 species in each mixture, but when they are combined, 8 species of these mixtures are the same as shown by the compositions in Methods. Thus, the combination of A, B and C gave 22 different species, all of which were separated with virtually baseline efficiency as shown in Figure 1. There are several critical pairs in this mixture. The 4-component critical pair mixture, PPP, PPO, POO and OOO, is marked out in Figure 1 as an example. These species have an ECN of 48 calculated on the basis that one double bond has the equal and opposite effect of 2 methylene groups. However, they obviously differ in their partition coefficients in order to be separated.

Mixture D also contained 10 species as shown in Figure 2. It was not added to the mixture of A, B and C because only 3 species, LnLnO, LnLo and LnOO, were different. However, mixture D is important for the identification of the species of soybean oil which contains triglycerides with acyl groups of linolenic and linoleic acid in appreciable amounts. The interesting feature of this reference mixture (Fig. 2) is that it contains 3 pairs of species that not only have the same ECN but the same number of carbon atoms and the same number of double bonds. Species LnLL and LnLnO (Fig. 2) have an ECN of 40, 54 carbon atoms and 7 double bonds. Species LLL and LnLO have an ECN of 42, 54 carbon atoms and 6 double bonds. Species LLO and LnOO have an ECN of 44, 54 carbon atoms and 5 double bonds. All 6 of these species as well as other species in this mixture were separated well enough to serve as reference compounds to determine their relative order of elution.

In order to demonstrate that a rapid analysis can be made with the FID using short, large bore columns, the 4-component critical pair mixture set out in Figure 1 was analyzed isocratically with 45% methylene chloride in acetonitrile as shown in the insert in Figure 2. These compounds emerged in approximately 2 min in a 15-min run, showing not only that fast eluting peaks can be analyzed with the FID, but that a rapid analysis can be performed with the methylene chloride-acetonitrile solvent system.

The plot of the response (peak area) vs mass of individual components of a standard mixture of simple triglycerides varying in unsaturation from 0 to 9 double bonds is shown in Figure 3. This plot gave a linear relationship satisfying the parameters for a quantitative analysis based on a proportionality of peak areas over the molecular weight range of species found in common vegetable oils. The linear dynamic range extended to approximately 200 μg, which enabled the analysis of minor components in concentrations of less than 1%. The table in Figure 3 shows that the relative error of the method for the analysis of minor components is approximately 7%, which decreases to approximately 1% for the major components.

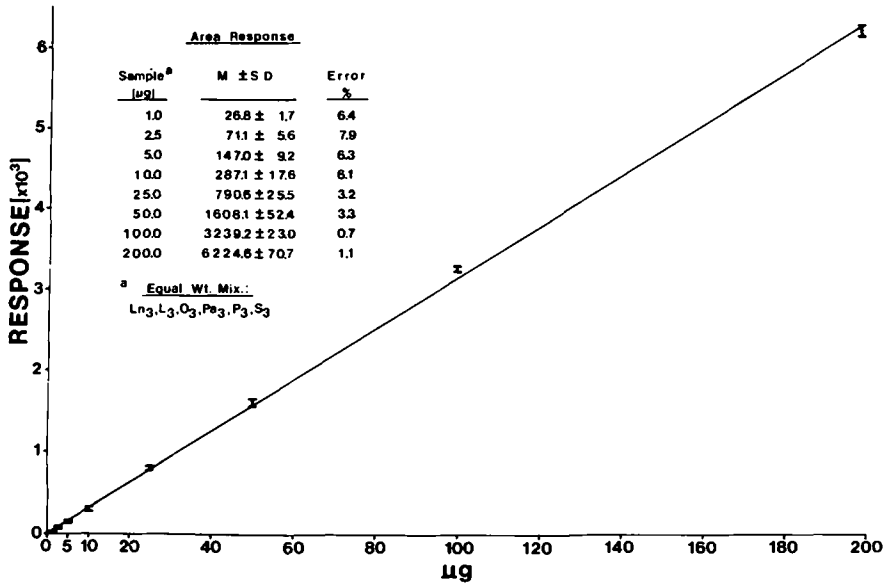

FIG. 3. A plot of the response versus μg injected for an equal weight mixture of triglycerides. Ln_3 = trilinolenin; L_3 = trilinolein; O_3 = triolein; Pa_3 = tripalmitolein; P_3 = tripalmitin; S_3 = tristearin. M ± SD = the mean ± standard deviation of the responses for each individual triglyceride in the mixture (n = 8).

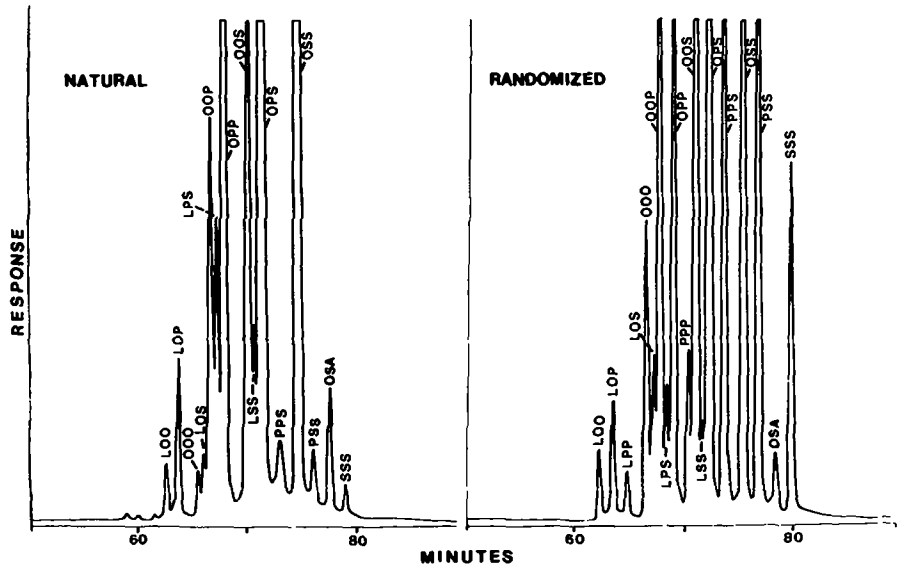

FIG. 4. Separation of natural and randomized cocoa butter triglyceride molecular species. Conditions and identification as in Fig. 1. A = arachidate.

TABLE 2

Analysis of Cocoa Butter

Molecular species[a]	Total calculated random (%)	HPLC natural (%)	Calculated 1,3 random 2 random (%)	HPLC total random (%)
LOO	1.5	0.6 ± 0.2[b]	0.4	1.1 ± 0.2[b]
LOP	2.2	1.7 ± 0.2	1.9	1.4 ± 0.2
OPaP	–	–	0.7	–
LPP	0.8	tr	1.1	0.6 ± 0.1
OOO	4.4	0.4 ± 0.1	0.8	3.8 ± 0.2
LOS	3.0	0.5 ± 0.1	2.5	1.9 ± 0.2
OOP	9.4	3.8 ± 0.1	6.3	9.8 ± 0.3
OPaS	0.4	–	0.9	–
LPS	2.1	2.9 ± 0.1	2.8	1.6 ± 0.2
OPP	6.6	14.6 ± 0.2	12.0	8.0 ± 0.2
PPP	1.6	tr	–	2.0 ± 0.1
OOS	13.0	5.8 ± 0.3	8.3	12.6 ± 0.6
LSS	1.5	1.3 ± 0.2	2.2	0.9 ± 0.1
OPS	18.3	38.5 ± 0.4	31.7	19.9 ± 0.6
PPS	6.4	0.5 ± 0.1	0.8	8.3 ± 0.5
OSS	12.6	26.0 ± 0.3	21.0	12.8 ± 0.3
PSS	8.9	0.6 ± 0.1	0.8	9.7 ± 0.2
OSA	0.7	1.1 ± 0.1	–	1.0 ± 0.4
SSS	4.1	0.3 ± 0.1	0.4	4.4 ± 0.1

[a]L = linoleate; O = oleate; Pa = palmitoleate; P = palmitate; S = stearate; A = arachidate (the order of designation does not indicate positional isomers).

[b]Mean ± S.D.

Identification of triglyceride species composition of natural oils was made by determination of the order of elution of all of the species that could be present in the oil by a modification of the computer program described by Merrit et al. (21) to take into account their theoretical carbon numbers (TCN). The peaks in the chromatogram of the oils are then identified by matching the calculated and the experimental TCN values relative to the order of elution of the components of reference mixtures of the same and similar species. The TCN values were determined according to El-Hamdy and Perkins (8) using constants for palmitoleate, oleate, linoleate and linolenate in reference mixtures under the specific chromatographic conditions employed. The calculated and experimental TCN values frequently will vary slightly, but

they are sufficiently reproducible to give the order of elution when compared with reference compounds. The order of elution and identification of the species in the oils examined here was fairly simple because virtually all of the species were present in the reference mixtures.

Application of this technique to identification of the species in natural and randomized cocoa butter is shown in Figure 4. All of the species except LOS, LPS and LSS were present in the reference mixtures. Quantitative analyses of the species of these oils are summarized in Table 2. The analyses in Table 2 show that in the natural sample, the distribution of the fatty acids among the triglycerides does not follow a random pattern but agrees in principle with a 1,3-random-2-random distribution proposed by Vander Wal (22). On the other hand, the analysis of the species of the randomized cocoa butter shows good agreement with that calculated for a random distribution which is further verification of the method.

The chromatogram of the analysis of soybean oil is shown in Figure 5. Identification of the species composition of this oil also was made by comparision of the retention times of the peaks relative to the order of elution of reference compounds and by their TCN values. All of the species in this oil, except LLS, LOS and LPS, were present in the reference mixtures A, B, C and D. It is generally believed that the triglyceride species of this oil conform to a random distribution pattern and the experimental values are in general agreement with this type of fatty acid distribution as shown in Figure 5.

Application of the method of identification to the molecular species of pure olive oil is shown in Figure 6. The triglyceride species composition of samples of pure olive oil from Italy and Spain as shown in Table 3 were virtually identical in spite of the fact they came from widely different geographical sources. These analyses show that olive oil (Table 3; Fig. 6) is characterized by 3 major components, LOO, OOO and OOP.

DISCUSSION

Although only 3 oils were analyzed, the results indicate that the FID used in the present study provides a quantitative analysis of the triglycerides of vegetable oils by HPLC insofar as they can be separated. The linear relationship of response to peak area showed good agreement for a variety of simple triglycerides which cover the elution time and equivalent carbon numbers of those found in common vegetable oils. With fats and oils other than those analyzed here, different reference mixtures might be used depending on their fatty acid composition.

The analyses of cocoa butter before and after randomization provide a good test of the method because the natural fat has a non-random triglyceride composition and randomization gives well-defined differences.

It has been well established that the triglyceride species compositions of both olive and soybean oils follow a random pattern. However, there were minor discrepancies in both cases although overall there was no doubt of the

Molecular Species	Calc. Rand. %	HPLC %	Molecular Species	Calc. Rand. %	HPLC %
LnLnL	0.8	1.3±0.3	LOP	7.6	8.4±0.4
LnLL	6.2	6.1±0.5	LnOS	0.4	–
LnLnO	0.3	tr	LPP	1.8	2.0±0.1
LLL	15.7	18.8±0.6	OOO	1.1	2.8±0.5
LnLO	5.2	5.3±0.9	LLA	0.5	–
LnLP	2.4	2.8±0.5	LOS	3.1	2.7±0.2
LLO	19.7	17.3±0.7	OOP	1.6	2.3±0.4
LnOO	1.1	tr	LPS	1.4	1.2±0.3
LLP	9.1	12.8±0.8	OPP	0.7	0.5±0.2
LnLS	1.0	–	LOA	0.4	–
LnOP	1.0	tr	OOS	0.6	0.8±0.2
LOO	8.2	7.6±0.3	LSS	0.3	–
LLS	3.7	3.9±0.1	OPS	0.6	0.6±0.1

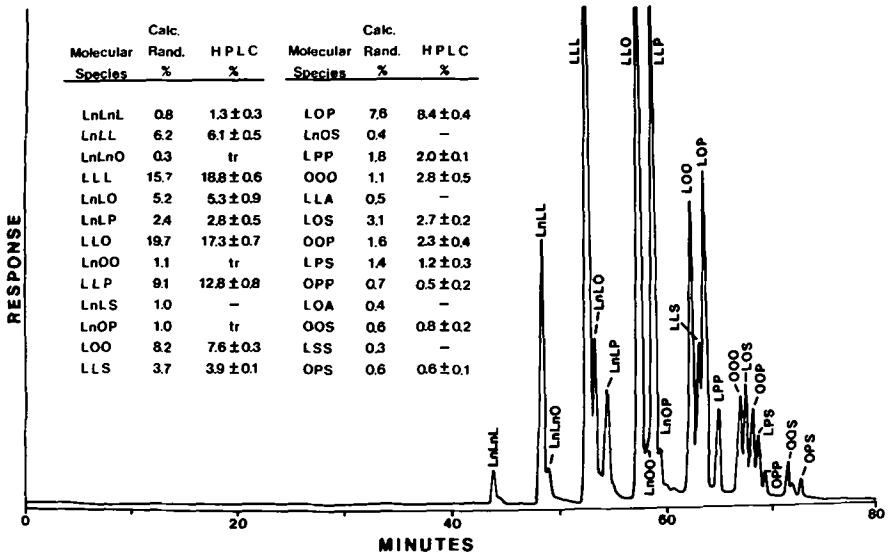

FIG. 5. Separation of soybean oil triglyceride molecular species. Conditions and identification as in Fig. 1.

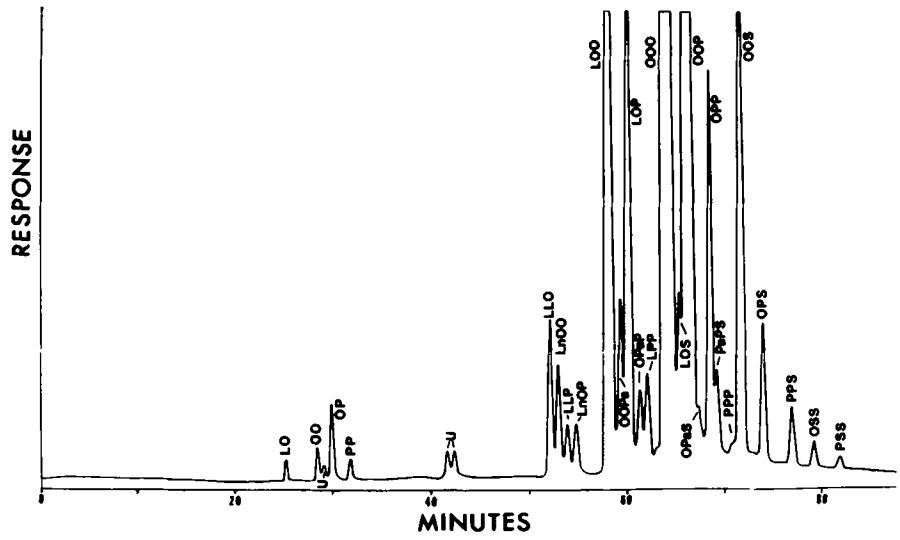

FIG. 6. Separation of pure Italian olive oil triglyceride molecular species. Conditions and identification as in Fig. 1.

TABLE 3

Analyses of Pure Olive Oils

Molecular Species	Italian		Spanish	
	Calculated Random (%)	HPLC (%)	Calculated Random (%)	HPLC (%)
LLO[a]	1.68	1.14 ± 0.02[b]	1.51	1.20 ± 0.07[b]
LnOO	1.19	0.91 ± 0.03	1.00	1.03 ± 0.02
LLP	0.27	0.38 ± 0.03	0.28	0.33 ± 0.03
LnOP	0.39	0.39 ± 0.03	0.37	0.39 ± 0.08
LOO	14.44	10.36 ± 0.09	13.16	9.80 ± 0.36
OOPa	1.70	1.18 ± 0.03	2.18	1.54 ± 0.03
LOP	4.71	4.91 ± 0.06	4.82	4.80 ± 0.11
OPaP	0.56	0.53 ± 0.02	0.80	0.70 ± 0.03
LPP	0.38	0.53 ± 0.04	0.44	0.86 ± 0.07
OOO	41.29	41.51 ± 0.30	38.33	38.51 ± 0.53
LOS	1.00	1.15 ± 0.15	1.24	1.34 ± 0.30
OOP	20.19	24.80 ± 0.25	21.07	26.13 ± 0.38
OPP	3.29	3.03 ± 0.07	3.86	3.40 ± 0.20
PPaS	–	0.55 ± 0.09	–	0.70 ± 0.09
OOS	4.29	3.98 ± 0.10	5.42	4.97 ± 0.10
OPS	1.40	0.85 ± 0.10	1.99	1.16 ± 0.03
PPS	0.11	0.47 ± 0.01	0.18	0.57 ± 0.02
OSS	0.22	0.19 ± 0.01	0.26	0.27 ± 0.01

[a]Ln - linolenate; L = linoleate; O = oleate; Pa = palmitoleate; P = palmitate; S = stearate (the order of designation does not indicate positional isomers).

[b]Mean ± S.D.

basic patterns. The question arises as to whether or not the experimental analyses represent the true composition and the calculated values an approximation inasmuch as the fatty acids are not expected to follow a true random pattern in natural fats (23).

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REFERENCES

1. Pei, P.T.S., Henry, R.S., and Ramachandran, S. (1975) Lipids 10, 152-156.
2. Plattner, R.D. (1981) in Methods in Enzymology (Lowenstein, J.M., ed.) Vol. 72: Lipids Part D,

Chapter 3, pp. 21-34, Academic Press, Inc., NY.
3. Herslof, B., Podlaha, O., and Toregard, B. (1979) J. Am. Oil Chem. Soc. 56, 864-866.
4. Schulte, E. (1981) Fette. Seifen. Anstrichm. 83, 289-291.
5. El-Hamdy, A.H., and Perkins, E.G. (1981) J. Am. Oil Chem. Soc. 58, 867-872.
6. Podlaha, O., and Toregard, B. (1982) J. High Resol. Chromatogr. Chromatogr. Commun. 5, 553-558.
7. Bezard, J.A., and Ouedraogo, M.A. (1980) J. Chromatogr. 196, 279-293.
8. El-Hamdy, A.H., and Perkins, E.G. (1981) J. Am. Oil Chem. Soc. 58, 49-53.
9. Dong, M.W., and DiCesare, J.L. (1983) J. Am. Oil Chem. Soc. 60, 788-791.
10. Wada, S., Koizumi, C., and Nonaka, J. (1977) Yukagaku 26, 95-99.
11. Privett, O.S., and Nutter, L.J. (1967) Lipids 2, 149-154.
12. Blank, M.L., Verdino, B., and Privett, O.S. (1965) J. Am. Oil Chem. Soc. 42, 87-90.
13. Blank, M.L., and Privett, O.S. (1964) J. Dairy Sci. 67, 481-488.
14. Singleton, J.A., and Pattee, H.W. (1984) J. Am. Oil Chem. Soc. 61, 761-766.
15. Perrin, J.-L., and Naudet, M. (1983) Rev. Fr. Corps Gras. 30, 279-285.
16. Phillips, F.D., Erdahl, W.L., Nadenicek, J.D., Nutter, L.J., Schmit, J.A., and Privett, O.S. (1984) Lipids 19, 142-150.
17. Privett, O.S., and Erdahl, W.L. (1978) Anal. Biochem. 84, 449-461.
18. Nickell, E.C., and Privett, O.S. (1967) Separ. Sci. 2, 307-318.
19. Christie, W.W. (1982) J. Lipid Res. 23, 1072-1075.
20. Mattson, F.H., and Volpenheim, R.A. (1961) J. Lipid Res. 2, 58-62.
21. Merritt, Jr., C., Vajdi, M., Kayser, S.G., Halliday, J.W., and Bazinet, M.L. (1982) J. Am. Oil Chem. Soc. 59, 422-432.
22. Vander Wal, R.J. (1960) J. Am. Oil Chem. Soc. 37, 18-20.
23. Blank, M.L., and Privett, O.S. (1966) Lipids 1, 27-30.

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Analysis of Sterol Esters by Capillary Gas Chromatography-Electron Impact and Chemical Ionization-Mass Spectrometry

WILLIAM R. LUSBY,* MALCOLM J. THOMPSON and **JAN KOCHANSKY,** *Insect Physiology Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Maryland 20705*

ABSTRACT

Synthetic mixtures of C_{40} to C_{47} sterol esters in groups of 7 esters were effectively separated and analyzed by capillary gas chromatography-mass spectrometry. Ammonia chemical ionization of all 20 sterol esters analyzed at a source block temperature of 120 C yielded $(M+NH_4)^+$ and $(M+H-RCO_2H)^+$ ions of high abundance or as base peak, thereby indirectly indicating the molecular weights of the ester and the sterol and acid moieties. Ammonia CI spectra of all esters containing a Δ^5-sterol moiety exhibited in addition to the above 2 ions an $M+NH_4-RCO_2H$ fragment. At a source block temperature of 150 C, $M+H-RCO_2H$ fragment was the base peak for all esters, and there was little or no indication of an $(M+NH_4)^+$ adduct ion. Protonated molecules were not observed for any esters analyzed by methane or isobutane CI. Molecular ions of 3-14% intensity were obtained for only 3 of the esters analyzed by electron impact; they contained a Δ^7-bond in the sterol nucleus, and the acid moiety was either saturated normal or branched chain or contained a single double bond. The base peak was a function of both the acid and sterol moieties of the sterol ester. The esters containing both saturated straight chain acid and saturated sterol moieties exhibited a base peak at m/z 215. The Δ^5-sterol esters with saturated branched or straight chain acid moieties exhibited base peaks at $M-RCO_2H$. Other ions also were of diagnostic value.

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INTRODUCTION

Direct gas chromatographic-mass spectrometric (GC-MS) analysis of sterol esters, compounds which are ubiquitous in both the plant and animal kingdom, has been hindered by gas-liquid chromatographic limitations of efficiently separating and eluting these high molecular weight compounds from the GC column as well as the inability to produce ions characteristic of the molecular weight (1,2,3). The development of on-column injectors and fused silica capillary columns together with bonded liquid phases has greatly increased the capability of GLC to analyze sterol esters (3,4).

Electron impact mass spectrometric analysis of sterol esters, via either direct probe or GC, has not provided ions directly indicative of the molecular weight. Methane has been used for chemical ionization mass spectrometry of sterol esters (3), and produced fragment ions which indicate both the acid and sterol moieties, but did not produce a molecular ion of the intact ester. The lack of ions representative of the molecular weight becomes a problem when samples are analyzed via direct probe or when 2 or more esters co-elute from a gas chromatograph. Isobutane chemical ionization (1) of sterol esters produced ions which again indicated the acid and sterol portions but not the intact molecule. The use of ammonia as a reagent gas has been reported to produce ammonium adduct ions $(M+NH_4)^+$, at low intensity in one study (1) and at high abundance in another study (5). None of the CI studies has investigated the influence on spectral characteristics of unsaturation in the acid or sterol moiety, and most studies have been limited to Δ^5-sterol esters.

We report here the development of EI and CI techniques for the GC/MS analysis of intact sterol esters, by which the latter produced ions indicative of the molecular weight as well as the sterol and acid portions of the molecule. Further, we report the effect of unsaturation at various sites in the acid and sterol moieties on the distribution of charge as well as the effect of reagent gas ion composition and ionization chamber temperature on spectral features.

EXPERIMENTAL PROCEDURES

Sterols and Fatty Acids

Sitosterol, stigmast-5-en-3β-ol (6), was prepared from stigmasterol by the method of Steele and Mosettig (7). Campesterol (campest-5-en-3β-ol) was obtained by fractional crystallization from acetone of soybean sterols from which the stigmasterol had been removed. Cholesterol, recrystallized once from ethanol, was obtained from Fisher Chemical Co. Isofucosterol [24-*trans*-stigmasta-5,24(28)-dien-3β-

*To whom correspondence should be addressed at Insect Physiology Laboratory, Bldg. 467, BARC-East, Beltsville, MD 20705.

ol] and 24-methylenecholest-5-en-3β-ol were obtained from rape pollen. 5α-Cholestan-3β-ol and 5α-campestan-3β-ol were prepared by the catalytic hydrogenation of cholesterol and campest-5-en-3β-ol, respectively. Similarly, 5α-campest-7-en-3β-ol and 5α-cholest-7-en-3β-ol were obtained via hydrogenation, in the presence of Raney nickel, of 7-dehydrocampesterol and 7-dehydrocholesterol, respectively. Campestively. Campesterol and campestanol were pesterol and campestanol were >95% purity, and the other sterols were >98% purity by GLC and TLC.

9,12-Octadecadienoic acid (linoleic acid), 9,12,15-octadecadienoic acid (linolenic acid), 9-hexadecenoic acid (palmitoleic acid), and 9-tetradecenoic acid (myristoleic acid) were purchased from Supelco, Bellefonte, Pennsylvania. The 4-methyl acids were prepared from 1-bromodecane or 1-bromododecane as follows. 2-Methyldodecanoic acid was prepared by the method of Allen and Kalm (8, note 2) and reduced with lithium aluminum hydride to give 2-methyldodecanol. Treatment with phosphorus tribromide/ether/pyridine gave the bromide, which was allowed to react with sodiomalonic ester under conditions analogous to those of the 2-methyldodecanoic acid preparation which gave, after saponification and decarboxylation, 4-methyltetradecanoic acid (mp 34-35 C after crystallization from hexane). 4-Methylhexadecanoic acid was prepared similarly except that 2-methyltetradecanol was converted to the methanesulfonate (methanesulfony chloride/pyridine) instead of the bromide for use in the malonic ester synthesis. The product melted at 44.5-45.5 C after crystallization from hexane.

Tridecanoic acid of 97% purity was purchased from Aldrich Chemical Co., Milwaukee, Wisconsin. All other acids were >98% purity by TLC and GLC analyses of their methyl esters.

Reagent Gases

Ammonia (99.99%), isobutane (99.5%), and methane (99.97%) were purchased from Matheson (Rutherford, New Jersey).

General Procedure for Preparation and Purification of Sterol Esters (Cholestan-3β-ol Tridecanoate as Model Compound)

Tridecanoic acid (17 mg) in 2 ml of benzene was converted to the acid chloride by refluxing with 0.02 ml of thionyl chloride (3 fold excess) in dry benzene for 4 hr. The benzene and excess thionyl chloride were removed under vacuum, then 25 mg of cholestan-3β-ol in 2 ml of benzene was added to the flask and the mixture was allowed to react with stirring overnight or in an oven at 50 C without stirring. The solvent was removed under vacuum, and the crude product was chromatographed over 5 g (10 mm × 65 mm) neutral alumina (activity grade II) and the following fractions were collected: a 50 ml fraction of hexane then two 25 ml fractions of benzene-hexane (1:1). The first 25 ml portion of benzene-hexane yielded about 20 mg of cholestan-3β-ol tridecanoate. A TLC analysis on a Silica Gel G plate in a solvent system of toluene-hexane (1:1) showed only one spot. The ester was used for mass spectral analyses without any further purification.

Instrumentation

Mass spectra were obtained using a Finnigan-MAT model 4510 gas chromatograph-mass spectrometer (GC-MS) fitted with a 15 meter by 0.25 mm fused silica capillary column coated (0.1 μm) with a bonded methyl silicone phase (DB-1, J&W, Inc.). Data were collected and analyzed via an Incos data system. In order to improve transmission of sample from the GC into the spectrometer ionizing chamber, the following 2 modifications were made. First, the separator thermal protect switch, initially set to activate at 280 C, was reset to activate at 375 C. Second, the moveable transfer line assembly was heated by the application of a heating tape (previously baked out at 410 C in a muffle oven). The temperature was monitored via an iron-constantan thermocouple arbitrarily placed between the heating tape and the transfer-line assembly. Sufficient current was supplied to the heating tape to cause a rise of thermocouple temperature from an initial value of 90 C to 185 C. For both electron impact and chemical ionization GC-MS spectra 7 or 8 sterol esters in 0.3 μl of hexane were introduced via on column injector (J&W 200-1020), and the column was held at 280 C for 1 minute, then temperature programmed to 350 C at a rate of 10 C/minute. Helium (Air Products, Allentown, Pennsylvania) was used as the carrier gas at a pressure of 20 psig.

Electron impact spectra were collected at 70 eV and a source block temperature of 150 C. Chemical ionization spectra were obtained at 2 source block temperatures of 150 C and 120 C for all compounds and at 100 C for a limited number of compounds. The indicated reagent gas pressures were: methane, 0.30 Torr; isobutane, 0.35 Torr, and ammonia, 0.60 Torr. Reagent gas ionic distribution was recorded at the beginning of each GC-CI-MS sample analysis by collecting spectra from 10 to 200 amu while operating the electron multiplier at reduced voltage. Reagent gas ion ratios were: methane (CH_5:H_3O:C_2H_5:C_3H_5), 150 C (100:24:84:22), 120 C (100:40:72:20); isobutane (NH_4(H_2O):

TABLE 1

Relative Retention Times of Sterol Esters[a]

	Sterol esters[b]	RRT
(I)	5α-Cholestan-3β-ol tridecanoate	1.00
(II)	Campest-5-en-3β-ol tridecanoate	1.17
(III)	Cholest-5-en-3β-ol 9-tetradecenoate	1.18
(IV)	Cholest-5-en-3β-ol 4-methyltetradecanoate	1.26
(V)	5α-Cholest-7-en-3β-ol 9-tetradecenoate	1.29
(VI)	5α-Cholest-7-en-3β-ol 4-methyltetradecanoate	1.39
(VII)	Stigmasta-5,24(28)-dien-3β-ol tridecanoate	1.41
(VIII)	24-Methylenecholest-5-en-3β-ol 4-methyltetradecanoate	1.50
(IX)	5α-Cholestan-3β-ol 9-hexadecenoate	1.71
(X)	5α-Cholestan-3β-ol 4-methylhexadecanoate	1.85
(XI)	5α-Cholest-7-en-3β-ol hexadecanoate	1.87
(XII)	24-Methylenecholest-5-en-3β-ol 9-hexadecenoate	2.00
(XIII)	5α-Cholestan-3β-ol 9,12,15-octadecatrienoate	2.49
(XIV)	5α-Cholest-7-en-3β-ol 9,12-octadecadienoate	2.71
(XV)	Campest-5-en-3β-ol 9,12,15-octadecatrienoate	3.00
(XVI)	5α-Campestan-3β-ol 9,12-octadecadienoate	3.02
(XVII)	5α-Campest-7-en-3β-ol 9,12,15-octadecatrienoate	3.34
(XVIII)	Stigmast-5-en-3β-ol 9,12-octadecadienoate	3.45
(XIX)	Stigmasta-5,24(28)-dien-3β-ol 9,12,15-octadecatrienoate	3.62
(XX)	Stigmasta-5,24(28)-dien-3β-ol 9,12-octadecadienoate	3.96

[a]Relative to 5α-cholestan-3β-ol tridecanoate (retention time 2.95 min) at 315 C on 15 m × 0.25 mm DB-1(0.1 μm) column with 20 psig of helium.

[b]Nomenclature according to Thompson et al. (6).

TABLE 2

Identification Numbers of Sterol Esters and Positions of Unsaturation in Sterol and Acid Moieties

Sterol moiety	Acid moiety				
			Position of unsaturation		
Position of unsaturation	Normal saturated	Branched saturated	Δ^9	$\Delta^{9,12}$	$\Delta^{9,12,15}$
Δ^0	I	X	IX	XVI	XIII
Δ^5	II	IV	III	XVIII	XV
Δ^7	XI	VI	V	XIV	XVII
$\Delta^{5,24(28)}$	VII	VIII	XII	XX	XIX

C_3H_3:C_3H_7:C_4H_9), 150 C (13:8:30:100), 120 C (40:12:20:100); ammonia (NH_4:$(NH_3)_2H$:$(NH_3)_3H$), 150 C (100:23:1), 120 C (100:34:3). The presence of m/z 19 (H_3O) remained in the methane reagent gas spectrum even after repeated purging. Similarly, m/z 18 (NH_4, or H_2O if residual electron impact was present), could not be removed from the isobutane reagent gas spectrum.

RESULTS

Twenty sterol esters that contained from 40 to 47 carbons, in groups of 7 esters, were successfully separated and eluted through the capillary GC and analyzed by GC/MS. Their relative retention times are given in Table 1. The results of mass spectral analyses are presented below. For convenience and immediate recognition of the basic structural features of sterol and acid moieties of sterol esters, we have prepared Table 2.

Electron Impact

Of the 20 sterol esters analyzed by electron impact, molecular ions of 3-14% intensity were obtained for only sterol esters V, VI and XI (Table 3). These 3 esters contained a Δ^7-bond in the sterol nucleus and the acid moiety was either branched or normal saturated, or contained only one double bond.

The base peak of sterol esters was a function of both the acid and sterol portion of the molecule (Table 3). The ester containing both saturated acid and sterol moieties exhibited a base peak at m/z 215, indicating loss of the acid

TABLE 3

Electron Impact Spectra of Sterol Esters

Esters[a]	M^+	M-RCOO	M-RCOOH	M-RCOOH $-CH_3$	M-RCOOH[b] -SC	M-RCOOH -SC-2H	M-RCOOH[c] -SC-D	Base peak	Other peaks
27[0]-13:0	584(0)	371(19)	370(54)	355(27)	257(13)	255(0)	215(94)	57	316(10)
27[0]-17BR	640(0)	371(21)	370(75)	355(27)	257(14)	255(1)	215(88)	57	316(12)
27[0]-16:1	624(0)	371(58)	370(24)	355(11)	257(13)	255(2)	215(28)	55	316(7)
28[0]-18:2	664(0)	385(27)	384(11)	369(3)	257(11)	255(0)	215(19)	81	330(2)
27[0]-18:3	648(0)	371(28)	370(3)	355(3)	257(4)	255(1)	215(11)	95	316(1)
28[5]-13:0	596(0)	383(33)	382(100)	367(14)	255(18)	253(1)	213(16)	382	
27[5]-15BR	610(0)	369(32)	368(100)	353(14)	255(16)	253(3)	213(14)	368	
27[5]-14:1	594(0)	369(33)	368(96)	353(18)	255(17)	253(0)	213(17)	55	
29[5]-18:2	676(0)	397(36)	396(58)	381(6)	255(12)	253(1)	213(13)	81	
28[5]-18:3	660(0)	383(37)	382(51)	367(5)	255(11)	253(0)	213(10)	81	
27[7]-16:0	624(12)	369(10)	368(10)	353(8)	255(73)	253(0)	213(31)	57	
27[7]-15BR	610(14)	369(12)	368(11)	353(9)	255(70)	253(0)	213(29)	57	
27[7]-14:1	594(3)	369(53)	368(12)	353(9)	255(38)	253(0)	213(23)	55	
27[7]-18:2	648(0)	369(57)	368(14)	353(4)	255(23)	253(2)	213(18)	95	
28[7]-18:3	660(0)	383(33)	382(9)	367(1)	255(19)	253(1)	213(15)	95	
29[5,24(28)]-13:0	608(0)	395(3)	394(10)	379(1)	255(3)	253(9)	213(10)	296	296(100)
28[5,24(28)]-15BR	622(0)	381(22)	380(68)	365(8)	255(8)	253(24)	213(16)	55	296(40)
28[5,24(28)]-16:1	634(0)	381(19)	380(48)	365(5)	255(8)	253(18)	213(12)	55	296(25)
29[5,24(28)]-18:2	674(0)	395(10)	394(8)	379(0)	255(3)	253(11)	213(13)	55	296(93)
29[5,24(28)]-18:3	672(0)	395(5)	394(2)	379(0)	255(0)	253(7)	213(10)	55	296(74)

[a]Sterol carbon number[site of unsaturation]-acid carbon number:degree of unsaturation. (BR = methyl branch on carbon number 4 of saturated acid moiety.)

[b]SC = Side Chain fragment from cleavage of carbon 17-20 bond.

[c]D = 42 mass units from fission of sterol ring D.

and side chain plus part of D-ring resulting from cleavage of the C-13, C-17 and C-14, C-15 bonds of the stanol nucleus together with the transfer of a hydrogen from the stanol nucleus (9). The abundance of this ion decreased when the acid moiety was either branched, mono-, di-, or tri-unsaturated (85, 28, 20, and 11%, respectively). The base peak for Δ^0-, Δ^5- or Δ^7-sterol esters containing di- and tri-unsaturated acid moieties was either at m/z 81 or 95 (C_6H_9 or C_7H_{11}) (Table 3). The Δ^5-sterol esters with saturated branched or straight chain acid moieties exhibited base peaks at M-RCO_2H (Table 3). The Δ^7- and $\Delta^{5,24(28)}$-sterol esters with saturated fatty acid moieties also gave prominent ions (but not base peaks) that correspond to M-RCO_2H, while for those containing an unsaturated acid group the prominent sterol ion occurred at M-RCO_2. The abundance of the ion corresponding to M-RCO_2H-CH_3 decreased with increasing unsaturation of either the sterol or acid moiety.

Fragments at m/z 257 and 255, common in the mass spectra of sterols containing saturated and Δ^5 or Δ^7 unsaturated nuclei, respectively (10,11), also were present in the mass spectra of the sterol esters with similar features. The monoene (257 amu) and diene (255) fragments, resulting from the loss of both the acid moiety and sterol side chain were approximately the same intensity for both saturated (4-14%) and Δ^5 compounds (10-18%), but significantly higher for Δ^7 compounds (19-74%). For the sterol ester with a $\Delta^{5,24(28)}$-sterol moiety the predominant steroid fragment occurred at m/z 253 and was presumably a steroid nucleus with 3 double bonds resulting from hydrogen transfer to the sterol side chain. The intensities of these fragments decreased with increased unsaturation of the acid portion of the molecule (Table 3).

Chemical Ionization

Methane. Protonated molecules were not observed for any esters analyzed by methane chemical ionization (CI). Methane CI of all the sterol esters, however, produced base peaks representative of the sterol moiety of the molecule. At a source block temperature of 150 C, the base peak for all esters containing an unsaturated sterol moiety corresponded to M+H-RCO_2H (Table 4) whereas for sterol

TABLE 4

Characteristic Ions From Methane Chemical Ionization GC-MS of Sterol Esters and the Effect of Unsaturation in Acid or Sterol Moieties on the Relative Abundance of These Ions

Sterol moiety		Acid moiety				
Position of unsaturation		Normal saturated	Branched saturated	Position of unsaturation Δ^9	$\Delta^{9,12}$	$\Delta^{9,12,15}$
			M-H-RCO_2H / M+H-RCO_2H (150 C)			
Δ^0		100/58	100/59	100/55	100/55	100/62
Δ^5		48/100	50/100	56/100	47/100	48/100
Δ^7		73/100	70/100	66/100	69/100	67/100
$\Delta^{5,24(28)}$		30/100	27/100	30/100	38/100	30/100
			M-H-RCO_2H / M+H-RCO_2H (120 C)			
Δ^0		100/100	100/92	100/92	100/90	100/100
Δ^5		34/100	36/100	37/100	31/100	32/100
Δ^7		47/100	42/100	44/100	45/100	44/100
$\Delta^{5,24(28)}$		25/100	22/100	20/100	23/100	22/100
			M-RCO_2H-CH_3 (150 C)			
Δ^0		32	33	31	35	34
Δ^5		21	29	22	22	25
Δ^7		26	28	25	24	23
$\Delta^{5,24(28)}$		10	11	12	11	9
			m/z 255 and 257 (150 C)			
	a.m.u.					
Δ^0	257	47	46	45	57	45
Δ^5	255	20	14	15	22	15
Δ^7	255	34	20	25	25	23
$\Delta^{5,24(28)}$	255	53	52	48	48	54

esters having a saturated sterol component, the base peak corresponded to M-H-RCO_2H. The abundance of M-H-RCO_2H for the unsaturated esters was related to the position of unsaturation of the sterol nucleus. For example, Δ^7-unsaturated compounds gave the highest abundance of M-H-RCO_2H (66-70%); Δ^5-compounds (48-56%); $\Delta^{5,24(28)}$ compounds (27-38%). Analysis of esters at a source block temperature of 120 C still produced base peaks representative of the sterol component though the intensities for M-H-RCO_2H were reduced (Table 4).

The abundance of M+H-RCO_2H-CH_4 of sterol esters at a source block temperature of 150 C also is related to both the degree of unsaturation and position of unsaturation of the sterol part (Table 4). The abundance of the $(M+H-RCO_2H-CH_4)^+$ ion is reduced at 120 C. The spectra of those esters containing C_{28} and C_{29} sterol moieties with $\Delta^{5,24(28)}$-bonds showed diagnostic ions at m/z 297 (40-64%) which correspond to M-RCO_2H-C_6H_{11} or C_7H_{13} which resulted from cleavage of the 22-23 bond. At 120 C the intensity of the ions at m/z 297 is reduced (8-14%). Spectra of esters having either saturated or $\Delta^{5,24(28)}$ sterol moieties indicated a greater abundance of low mass ions than did those of esters possessing a sterol part with a Δ^5- or Δ^7-bond.

The abundance of ions at m/z 257 and 255 from methane chemical ionization of sterol esters was related to the position and degree of unsaturation of the sterol component. Esters with saturated sterol moieties or sterol moieties containing $\Delta^{5,24(28)}$-bonds provided an abundance of approximately 50%, whereas the averages for esters containing sterol components with a Δ^7- or Δ^5-bond were 27 and 17%, respectively (Table 4).

Isobutane. Isobutane CI of sterol esters at 150 C did not produce protonated molecules but produced M+H-RCO_2H as the base peak for all esters regardless of the degree of unsaturation (Table 5). This is in contrast to methane CI where esters containing saturated sterol components produced M-H-RCO_2H as the base peak. For isobutane, however, the abundance of M-H-RCO_2H, except for compound I, was always greater than 50% for esters with saturated moieties. For esters with unsaturation in the sterol part, the abundance of this ion was always less than 36%.

Isobutane CI of esters bearing a saturated or Δ^7-sterol moiety gave a greater abundance of M-H-RCO_2H with increasing degree of unsaturation in the acid portion of the molecule. With Δ^5- and $\Delta^{5,24(28)}$-sterol compounds, this was not the case. The abundance of M-H-RCO_2H was qualitatively the same at 120 C, but the intensities were reduced. In contrast to methane CI, neither ions at m/z 257 and 255 nor the $(M-RCO_2H-CH_4)^+$ ions were observed in isobutane CI spectra. The ion at m/z 297, indicative of sterol ester with a sterol moiety having $\Delta^{5,24(28)}$-olefinic bonds, was present in lower (11-32%, 150 C) in isobutane CI spectra compared to methane CI spectra (40-64%, 150 C). The abundance of this ion was reduced to 1-11% at a lower source temperature of 120 C.

Ions diagnostic for the acid portions of the esters, $(RCO_2H-H)^+$, were of approximately the same abundance (4-66%) as for those produced by methane (8-50%). Interestingly, the abundance of the $(RCO_2H-H)^+$ (Table 5) ion was always greater than the abundance of the $(RCO_2H+H)^+$ ion for esters containing a branched saturated acid component; this relationship was always reversed for esters containing normal saturated or unsaturated acid moieties. This relationship was observed at both 120 and 150 C. Esters possessing both an unsaturated sterol portion and an acid moiety with 2 or 3 olefinic bonds yielded very low intensities for $(RCO_2H+H)^+$ and $(RCO_2H-H)^+$ ions. These ions were not of diagnostic value for the esters.

Ammonia. Only ammonia CI of sterol esters afforded ions indicative of the molecular weights of the sterol esters, sterol and acid moieties (Fig. 1). In fact, ammonia CI of the sterol esters at a source block temperature of 120 C yielded an $(M+NH_4)^+$ adduct ion as the base peak for all esters except for compounds IV, cholest-5-en-3β-ol 4-methyltetradecanoate (base peak was M+H-RCO_2H) and VIII, 24-methylenecholest-5-en-3β-ol 4-methyltetradecanoate (base peak was M+NH_4-RCO_2H) (Table 6). The $(M+NH_4)^+$ adduct ions for compound IV and VIII, however, were 40 and 60% relative abundances, respectively. Interestingly, ammonia CI spectra of all esters containing a sterol moiety with a Δ^5-bond exhibited, in addition to the $(M+NH_4)^+$ and $(M+H-RCO_2H)^+$ ions, an ion numerically equivalent to M+NH_4-RCO_2H which is coincidentally equivalent to the molecular ion of the sterol moiety (Fig. 2, Table 6). This feature readily distinguishes a Δ^5-sterol ester from a Δ^0- or Δ^7-sterol ester. At a source block temperature of 150 C, M+H-RCO_2H was the base peak for all esters (Fig. 3). Examination of a limited number of esters at a source temperature of 100 C produced $(M+NH_4)^+$ adduct ions as base peak; however, no other ions had an abundance greater than 10% (Fig. 4). Thus, the ammonia CI spectra of sterol esters obtained at a source temperature of 120 C were far more informa-

TABLE 5

Characteristic Ions From Isobutane Chemical Ionization GC-MS of Sterol Esters and the Effects of Unsaturation in Acid or Sterol Moieties on the Relative Abundance of These Ions

Sterol moiety	Acid moiety				
Position of unsaturation	Normal saturated	Branched saturated	Position of unsaturation		
			Δ^{9}	$\Delta^{9,12}$	$\Delta^{9,12,15}$
		M-H-RCO_2H / M+H-RCO_2H (150 C)			
Δ^{0}	35/100	55/100	85/100	94/100	68/100
Δ^{5}	18/100	18/100	18/100	13/100	16/100
Δ^{7}	24/100	21/100	20/100	36/100	36/100
$\Delta^{5,24(28)}$	18/100	9/100	8/100	8/100	11/100
		M-H-RCO_2H / M+H-RCO_2H (120 C)			
Δ^{0}	30/100	49/100	51/100	72/100	55/100
Δ^{5}	11/100	11/100	10/100	12/100	11/100
Δ^{7}	15/100	18/100	13/100	28/100	27/100
$\Delta^{5,24(28)}$	8/100	5/100	9/100	8/100	7/100
		RCO_2H+H / RCO_2H-H (150 C)			
Δ^{0}	60/30	28/40	42/4	51/13	66/10
Δ^{5}	15/11	14/24	14/6	5/2	7/3
Δ^{7}	11/9	20/28	22/0	12/4	6/3
$\Delta^{5,24(28)}$	25/22	14/30	23/8	10/7	13/5
		RCO_2H+H / RCO_2H-H (120 C)			
Δ^{0}	14/14	11/19	24/4	32/3	41/2
Δ^{5}	12/7	7/16	7/0	4/1	3/1
Δ^{7}	6/2	10/29	1/1	8/1	1/1
$\Delta^{5,24(28)}$	23/13	15/30	30/8	6/3	7/2

tive than any of the other CI spectra.

The ion representing RCO_2H+NH_4 was present in the spectra of many esters having an unsaturated acid portion, whereas many spectra of esters containing a saturated straight or branched chain acid group provided a diagnostic ion corresponding to $RCO_2H+NH_4-H_2$ (Table 6).

DISCUSSION

Through the use of an on-column injector, a fused silica capillary column coated with a bonded methyl silicone phase (DB-1), and a modified GC system which improved transmission of sample from the GC into the spectrometer ionizing chamber, we have successfully separated and analyzed a series of sterol esters by GC/MS using both electron impact and chemical ionization.

Electron Impact

Although molecular ions were obtained for only 3 of the sterol esters analyzed by GC/MS, certain other ions in the spectra were of diagnostic value in establishing the general structural features of the sterol moieties of the esters as well as indicating whether the fatty acid moieties were normal or branched saturated chain or normal unsaturated chain. For example, as with many Δ^5-sterol acetates (10, 12), the $(M-RCO_2H)^+$ ion was the base peak of a Δ^5-sterol ester containing branched or normal saturated chain or normal mono-olefinic acid moiety (Table 3). This peak readily establishes whether the ester contains a C_{27}, C_{28}, or C_{29} sterol moiety. Only the $\Delta^{5,24(28)}$-sterol esters containing normal chain acid moieties gave ions at m/z 296 as base peaks ($M-RCO_2H-C_6H_{12}$, for C_{28} sterols or $-C_7H_{14}$ for C_{29} sterols), whereas corresponding esters containing saturated branch chain or unsaturated acid moieties did not. The Δ^5-sterol esters containing unsaturated acid moieties gave as base peaks fragments from cleavage of the acid portions (Table 3). While the Δ^7- and $\Delta^{5,24(28)}$-sterol esters containing saturated acid moieties gave prominent $M-RCO_2H$ fragments, those containing unsaturated acids gave prominent ions corresponding to $M-RCO_2$. Whether these characteristics are common to other esters containing sterol or fatty acid moieties with double bond(s) present in other positions in their molecules remains to

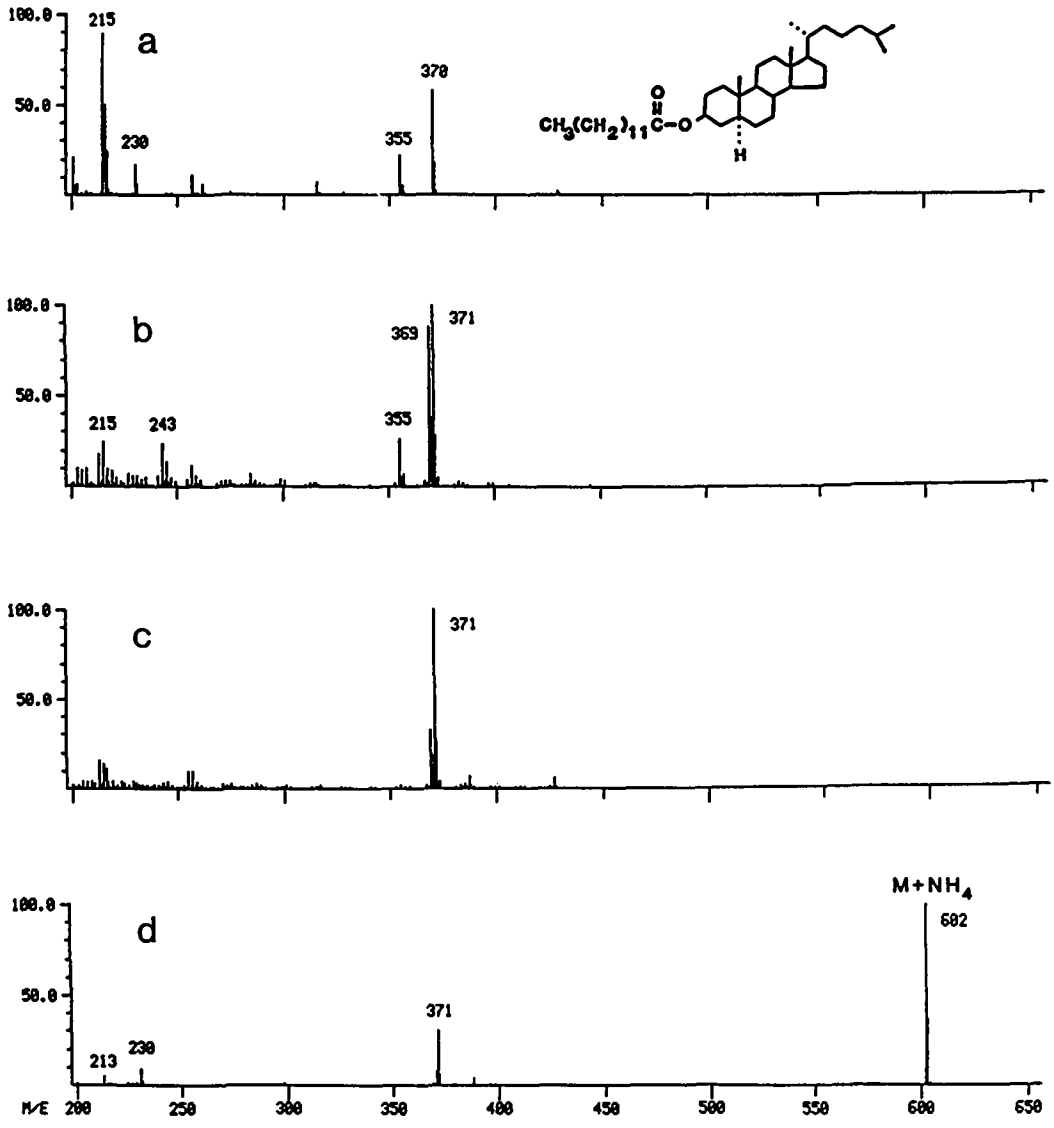

FIG. 1. GC-MS of 5α-cholestan-3β-ol tridecanoate at a source temperature of 120 C: (a) electron impact; (b) methane chemical ionization; (c) isobutane chemical ionization; (d) ammonia chemical ionization.

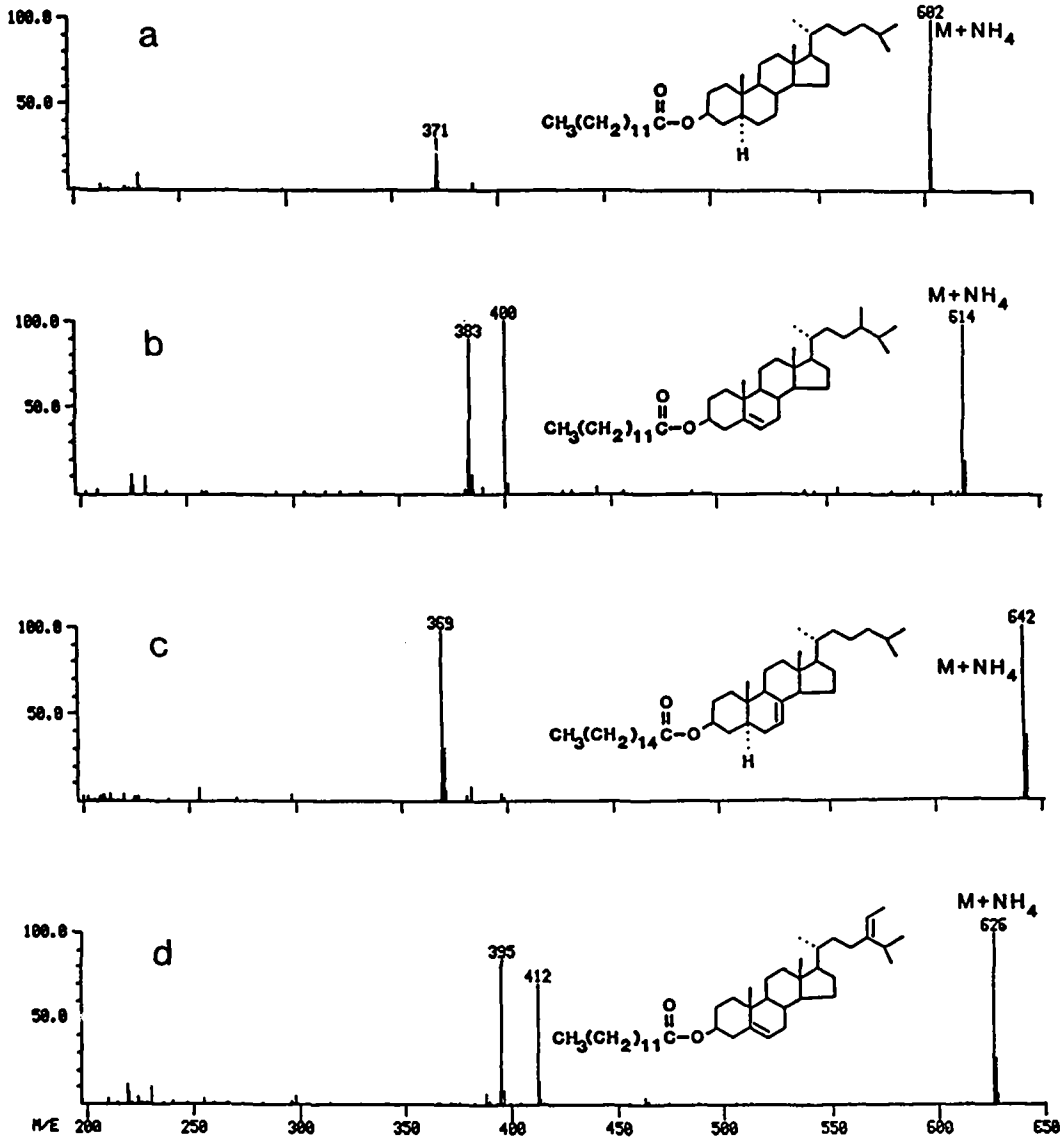

FIG. 2. Ammonia chemical ionization GC-MS at 120 C: (a) 5α-cholestan-3β-ol tridecanoate; (b) campest-5-en-3β-ol tridecanoate; (c) 5α-cholest-7-en-3β-ol hexadecanoate; (d) stigmasta-5,24(28)-dien-3β-ol tridecanoate.

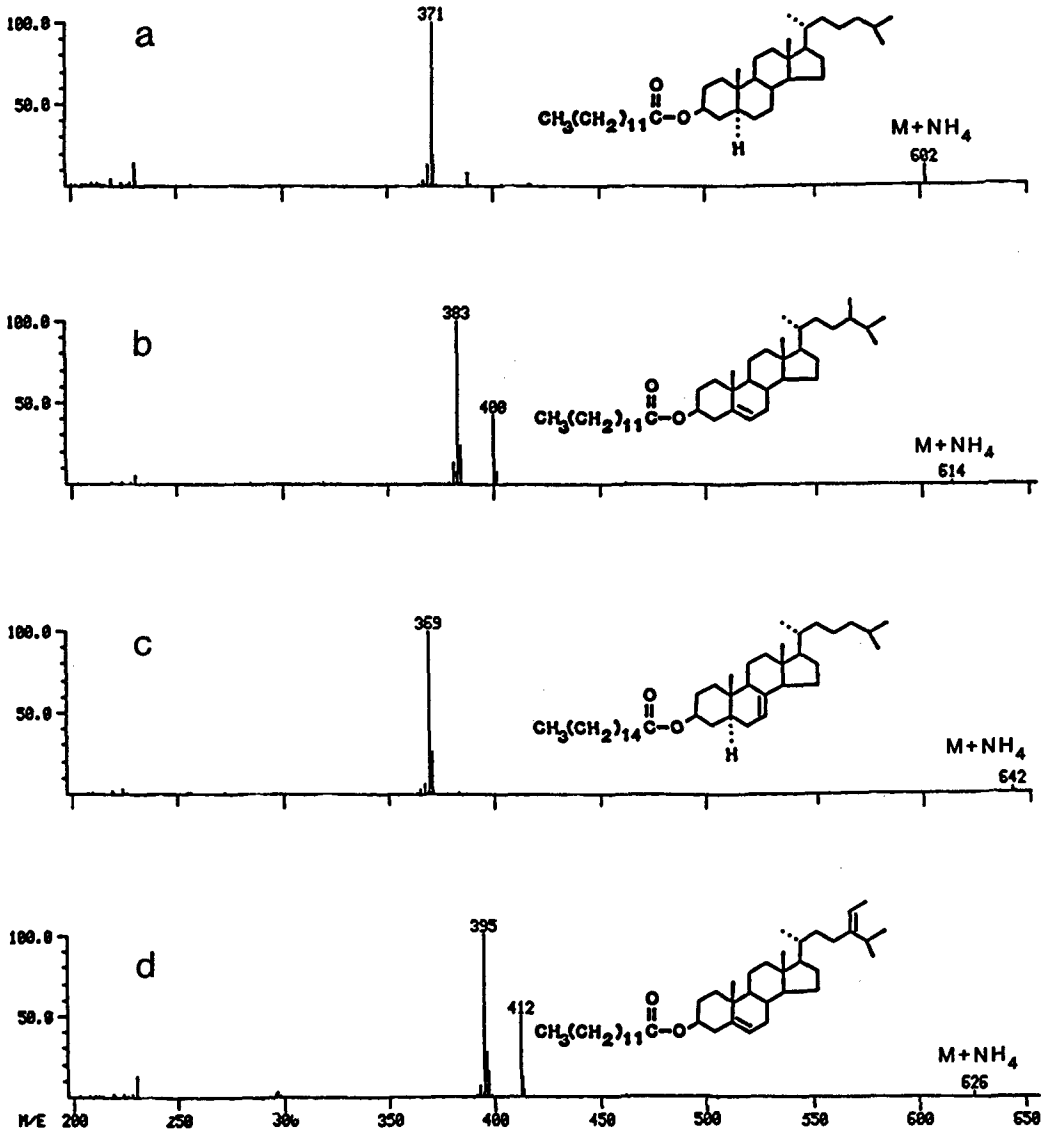

FIG. 3. Ammonia chemical ionization GC-MS at 150 C: (a) 5α-cholestan-3β-ol tridecanoate; (b) campest-5-en-3β-ol tridecanoate; (c) 5α-cholest-7-en-3β-ol tridecanoate; (d) stigmasta-5,24(28)-dien-3β-ol tridecanoate.

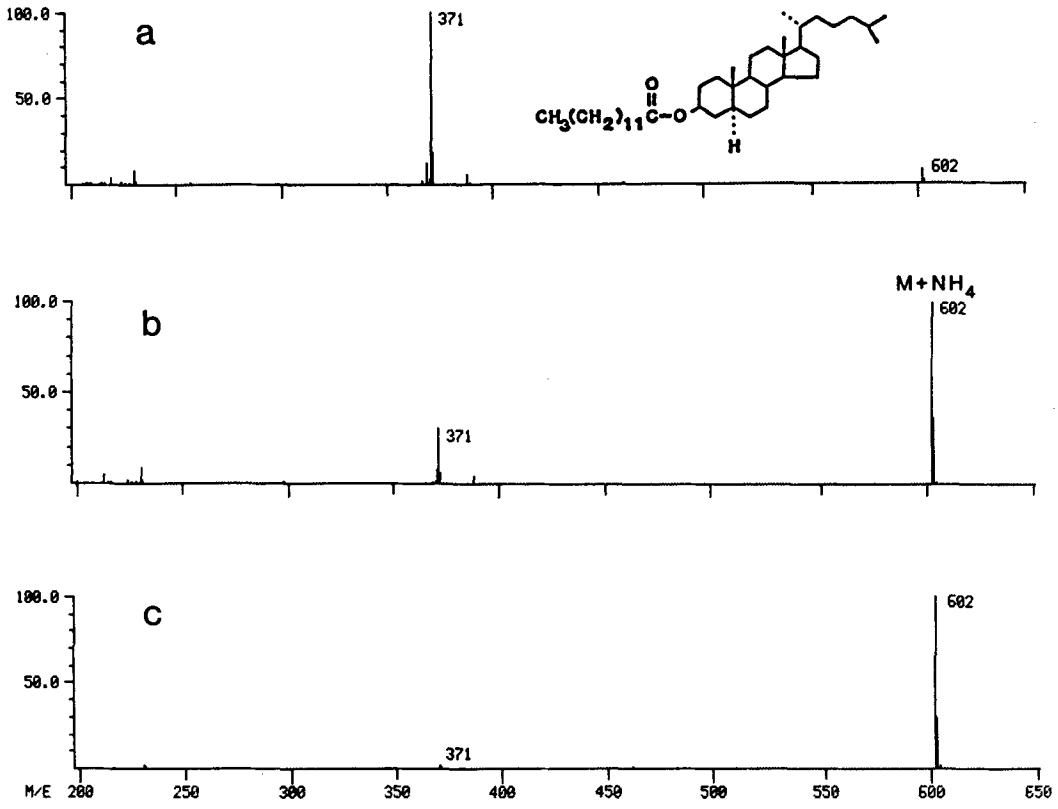

FIG. 4. Ammonia chemical ionization GC-MS of 5α-cholestan-3β-ol tridecanoate: (a) 150 C; (b) 120 C; (c) 100 C.

be determined. Esters containing both saturated acid and steroi moieties are readily distinguished from other sterol esters by exhibiting base peaks at m/z 215. Clearly, the foregoing results of GC/MS analyses of sterol esters indicate that considerable information can be acquired as to the basic structure of the sterol moiety as well as whether the acid moiety is either straight or branched chain and saturated or straight or branched chain and unsaturated.

Chemical Ionization

In chemical ionization studies of organic compounds it has been a common practice to report only the pressure of the gas used. There are several problems with using only this measurement to specify chemical ionization conditions. First, location of the pressure measuring device is, to varying degrees, remote from the actual site of ion production. Second, the location varies from instrument to instrument. Third, establishment of the same apparent gas pressure does not insure the same reagent gas ionic distribution. This may be due to differences in rate of air leaking into the instrument, variation of ion residence time within source due to different source geometries, water being desorbed from the wall of both reagent gas tubing and the spectrometer, and possibly residual reagent gas of a different type being desorbed from the walls of the gas lines and spectrometer. In this study we have obtained distinctly different CI spectra with the same compound at the same indicated reagent gas pressure. Examination of the actual ionic composition of the reagent gas, however, revealed significant differences. We also have obtained similar sample spectra with different gas pressures but with similar ionic distribution for the reagent gas. Thus, in order to more completely characterize the chemical ionization conditions, we suggest reporting the gas pressure, ionic distribution of the reagent gas and the source block temperature.

For example, ammonia chemical ionization experiments at a temperature of 120 C and at source pressure of 0.3, 0.6 and 0.9 Torr demonstrated dramatic changes in both reagent gas ionic distribution and sample ions produced. When the pressure was increased from 0.3 to

TABLE 6

Characteristic Ions From Ammonia Chemical Ionization GC-MS of Sterol Esters and the Effects of Unsaturation in Acid or Sterol Moieties on the Relative Abundance of These Ions

Sterol moiety	Acid moiety				
Position of unsaturation	Normal saturated	Branched saturated	Position of unsaturation		
			Δ^9	$\Delta^{9,12}$	$\Delta^{9,12,15}$
		$M+NH_4$ (150 C / 120 C)			
Δ^0	14/100	3/100	21/100	66/100	65/100
Δ^5	1/100	1/40	7/100	10/100	15/100
Δ^7	3/100	5/100	13/100	22/100	27/100
$\Delta^{5,24(28)}$	3/100	1/60	7/100	15/100	20/100
		$M+NH_4-RCO_2H$ (150 C / 120 C)			
Δ^0	8/0	11/8	7/1	2/1	8/1
Δ^5	43/100	45/83	41/67	32/23	32/30
Δ^7	1/1	1/1	1/1	1/1	1/1
$\Delta^{5,24(28)}$	49/69	51/100	49/74	40/21	38/29
		RCO_2H+NH_4 / $RCO_2H+NH_4-H_2$ (150 C)			
Δ^0	1/15	0/7	10/1	67/2	13/0
Δ^5	1/8	1/8	1/1	4/0	2/0
Δ^7	0/0	0/10	2/1	6/0	3/0
$\Delta^{5,24(28)}$	1/15	1/8	1/1	12/8	8/0
		RCO_2H+NH_4 / $RCO_2H+NH_4-H_2$ (120 C)			
Δ^0	0/10	1/5	2/0	31/1	14/0
Δ^5	2/12	1/23	2/1	18/2	19/1
Δ^7	1/3	4/40	3/1	15/1	0/0
$\Delta^{5,24(28)}$	2/13	0/21	14/3	21/3	37/0

0.9 Torr, the relative abundance of the ammonium ion decreased from 100% to 85% while the relative abundances of the reagent gas cluster ions $[(NH_3)_2H]^+$ and $[(NH_3)_3H]^+$ increased respectively from 21% to 100% and <1% to 43%. The above change in pressure increased the relative abundance of the ammonium adduct of the sample from 9% to 100%, while abundance of the ion indicative of the sterol moiety decreased from 100% to 11%.

Of the 3 reagent gases used in the chemical ionization GC/MS analysis of sterol esters, methane produced the greatest number of fragments. Isobutane generated fewer fragments than methane and ammonia even fewer than isobutane. This would be expected, however, from the proton affinities of methane, isobutane and ammonia of 127 kcal/mole, 195 kcal/mole and 207 kcal/mole, respectively.

At a source block temperature of 150 C, methane CI of all sterol esters containing an unsaturated sterol moiety gave a base peak that corresponded to $M+H-RCO_2H$, whereas for esters having a saturated sterol moiety the base peak corresponded to $M-H-RCO_2H$ (Table 4). An analogous ion, $(M-H-ROH)^+$, has been reported for the ammonia desorption chemical ionization analysis of saturated steryl benzyl ether (13). This is similar to the production of ions of $(M-H)^+$ for saturated hydrocarbons and $(M+H)^+$ for unsaturated hydrocarbons when analyzed by methane CI (15). The reduction of intensities for $M-H-RCO_2H$ at source temperature of 120 C suggests that this process is thermally driven and may correspond to $M+H-RCO_2H-H_2$, or alternatively the simple thermal elimination of an acid molecule (9) from the neutral ester followed by hydride abstraction by the ethyl reagent gas ion. Isobutane CI at 150 C of all esters produced $M+H-RCO_2H$ as the base peak regardless of the degree of unsaturation.

The ion $(M+H-RCO_2H)^+$ of strong intensity has been reported for methane CI of esters saturated in both the acid and sterol portions at a source pressure of 0.9 Torr and a temperature of 160 C (3). However, the intensity of the $M-H-RCO_2H$ fragment or the ionic distribution of the reagent gas was not reported.

The presence and absence of $M+H-RCO_2H-CH_4$ in methane and isobutane CI spectra respectively is related to the proton affinity (PA)

of the gases. Methane (PA = 127 kcal/mole) transfers sufficient energy to consecutively eliminate both RCO_2H and CH_4, whereas for isobutane (PA = 195 kcal/mole) only RCO_2H is eliminated.

Of the 3 reagent gases, ammonia CI of the sterol esters at source temperature of 120 C afforded the most basic and definitive information as to the molecular weights of the sterol esters, and of the sterol and acid moieties (Fig. 1). It is the method of choice for mass spectral analysis of sterol esters. At 120 C, ammonia CI yielded an $(M+NH_4)^+$ adduct ion as the base peak for all esters except for 2, and for those compounds adduct ions were of high relative abundance. In addition, at this source temperature the presence of a large $(M+NH_4-RCO_2H)^+$ ion which was observed only in esters containing a Δ^5-sterol moiety, immediately characterizes the sterol portion as a Δ^5-sterol (Fig. 2, Table 6). Similarly, $(M+NH_4-RCO_2H)^+$ ions were observed by Tecon et al. in ammonia desorption chemical ionization spectra of acetates and benzoates of both Δ^4 and Δ^5 cholestenols, but were not found in the spectra of acetates and benzoates of cholestanol (14). The authors concluded that one of the prerequisites for the formation of this ion or its stabilization seems to be a double bond in the neighborhood of the substitution center (14). We have observed a similar stabilizing effect in the ammonia chemical ionization spectra of esters of the allylic alcohols phytol and geranylgeraniol but not with the saturated dihydrophytol (16). Our success in obtaining relatively large $(M+NH_4)^+$ adduct ions and $(M+H-RCO_2H)^+$ ions for sterol esters is due to the operation of the mass spectrometer at a lower source block temperature (120 C) than that routinely used in mass spectral analysis. Certainly, the weak peaks for this adduct ion in the ammonia CI spectra of sterol esters obtained at a source block temperature 150 C (Fig. 3) supports this conclusion. Lowering the source temperature to 100 C still gives the $(M+NH_4)^+$ adduct ions as the base peak for sterol esters. However, the $(M+H-RCO_2H)^+$ ion which allows one to determine indirectly the molecular weights of the acid and sterol moieties is barely discernible (Fig. 4). Thus, the temperature of 120 C does appear to approach the ideal source temperature for obtaining the most useful information when analyzing sterol esters by ammonia CI.

While the reduction in source block temperature alters the ionic distribution of the reagent gas and thereby influences the rate of formation of $(M+NH_4)^+$ ions, it is likely that the instability of the $M+NH_4$ adduct of sterol esters causes a reduction in the relative intensity at elevated temperatures. Mass spectral analyses of organic compounds at low source block temperature certainly accelerate contamination of the source. However, with the advent of instruments that allow for readily changed ion volumes, the problems of source contamination and memory effects are greatly reduced.

The high resolution capabilities of capillary GC permit the separation and analyses by EI and CI of mixtures of esters that are different by only one carbon unit and in some instances simply by the addition and/or position of a double bond; hence the practical potential of analyzing complex mixtures of sterol esters by this method. In fact, in research concerned with the isolation of feeding stimulant(s) of the cotton boll weevil from cotton buds, we have successfully analyzed by GC-CI-MS a mixture of wax esters and sterol esters that was not separable by any other method (16). Furthermore, the combined use of electron impact and chemical ionization GC-MS has permitted us to obtain the molecular weights of these waxes and sterol esters, to identify nearly all of the alcohol and sterol moieties of waxes and sterol esters, respectively, of the cotton buds, and have allowed us to derive the basic structure of the acid moieties of these compounds. Being able to identify the esters demonstrates the advantages of this technique.

Certainly, the resolution capabilities of high temperature gas capillary columns have made it possible to analyze sterol esters by GC-MS with only minor modifications to the mass spectrometer. The exploitation of both electron impact and chemical ionization GC/MS as a tool for the analysis of waxes and sterol esters is limited only by the innovative ideas of the investigator.

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REFERENCES

1. Murata, T., Takahashi, S., and Takeda, T. (1975) Anal. Chem. 47, 577-580.
2. Smith, N.B. (1983) J. Chrom. 254, 195-202.
3. Wakeham, S.G., and Frew, N.M. (1982) Lipids 17, 831-843.
4. Smith, N.B. (1982) Lipids 17, 464-468.
5. Smith, L.L., Teng, J.I., Lin, Y.Y., Seitz, P.K., and McGehee, M.F. (1981) J. Steroid Biochem. 14, 889-900.
6. Thompson, R.H., Patterson, G., Thompson, M.J., and Slover, H.T. (1981) Lipids 16, 694-699.
7. Steele, J.A., and Mosettig, E. (1962) J. Org. Chem. 28, 571-572.
8. Allen, C.F., and Kalm, M.J. (1963) in Organic Syntheses collective volume IV (Rabjohn, N., ed.), pp. 618-619, John Wiley & Sons, Inc., New York.

9. Budzikiewicz, H., Djerassi, C., and Williams, D.H. (1964) Structural Elucidation of Natural Products by Mass Spectrometry, Holden-Day, Inc., San Francisco.
10. Heller, S.R., and Milne, G.W.A. (1978) EPA/NIH Mass Spectral Data Base, U.S. Government Printing Office, Washington, D.C.
11. Zaretskii, Z.V. (1976) Mass Spectrometry of Steroids, John Wiley & Sons, New York.
12. Knights, B.A. (1967) J. Gas Chrom., 273-282.
13. Hirano, Y., Djerassi, C., and Tecon, P. (1982) Abstracts 30th Ann. Confer. on Mass Spectrom. and Allied Topics, 143-144.
14. Tecon, P., Hirano, Y., and Djerassi, C. (1982) Org. Mass Spectrom. 17, 277-285.
15. Field, F.H. (1968) J. Am. Chem. Soc. 90, 5649-5656.
16. McKibben, G.H., Thompson, M.J., Parrott, W.L.; Thompson, A.C., and Lusby, W.R., manuscript in preparation.

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COMMUNICATIONS

Effect of Protein Depletion on the VLDL Triacylglycerol Secretion and Apoprotein Synthesis by the Perfused Liver from Pregnant Rats

H. TOURNIER, G. YARYOUR, O. MERCURI* and M.E. DE TOMÁS,* *Instituto de Investigaciones Bioquímicas de la Plata (INIBIOLP), Facultad de Ciencias Médicas, UNLP-CONICET, calles 60 y 120, 1900 - La Plata, Argentina.*

ABSTRACT

The effect of protein depletion in the pregnant rat on the polyunsaturated fatty acid incorporation into very low density lipoproteins (VLDL) has been investigated. The apoprotein pattern of these particles was determined. In in vivo experiments the amounts of serum and liver triacylglycerol were determined. VLDL were isolated and their apo C concentration calculated. In in vitro experiments the radioactivity of [^{3}H] leucine incorporated into VLDL apoproteins was measured. The results show that protein depletion during pregnancy promotes a drastic increase in serum and liver triacylglycerol. The VLDL isolated from these animals show an increase in the triacylglycerol/protein ratio and a decrease in their content of apo C. Meanwhile, a significant reduction in the [^{3}H] leucine incorporation into apo C peptides by the perfused liver of protein depleted rats was detected. On the other hand, protein deprivation did not affect labeled linoleic and arachidonic acid incorporation into triacylglycerol of the newly secreted VLDL. Taking these results together, let us deduce that a defective VLDL is secreted by the liver of the protein depleted pregnant rats. The abnormal composition of these particles may influence its normal metabolism through their effects on lipoprotein lipase and this fact could affect the normal supply of polyunsaturated fatty acids to the fetus. *Lipids* 19:902-905, 1984.

INTRODUCTION

The importance of an adequate supply of essential fatty acids (EFA) for normal fetal growth and tissue development is well-documented (1). Linoleic acid is the principal dietary form of EFA, and arachidonic acid is the principal functional form of EFA in mammalian tissues. The transformation of linoleic acid into arachidonic acid is limited by the in vivo activity of the enzyme $\Delta 6$ desaturate (2), and no measurable activity of this enzyme was detected in fetal rat liver and placenta (3). These results suggest that fetal arachidonic acid originates in the maternal liver from dietary linoleic acid. This assumption was confirmed by Pascaud et al. (4).

More recently, Mercuri et al. (3) demonstrated that the activity of liver $\Delta 6$ desaturase was profoundly affected by protein deprivation during pregnancy in the rat and, consequently, the normal supply of polyunsaturated fatty acids to the fetus could be altered.

No data are available in the literature at present on the effect of protein depletion in the transfer of polyunsaturated fatty acids from the mother's liver to the fetus. Hummel et al. (5) demonstrated that triacylglycerol from very low-density lipoproteins (VLDL) is a direct source of fetal fatty acids via lipoprotein lipase (LPL).

The present experiments were designed to study the effect of maternal protein depletion on the linoleic acid and arachidonic acid incorporation into VLDL triacylglycerol and on the apoprotein pattern of these particles which could affect their normal metabolism via lipoprotein lipase. This study is part of a continuous investigation of the influence of maternal protein deficiency on the supply of EFA to the fetus.

MATERIALS AND METHODS

[1-^{14}C] linoleic acid, specific activity 52 mCi/mmol, radiochemical purity 98%, arachidonic acid 5,6,8,9,11,12,14,15-^{3}H (N) specific activity 62.2 Ci/mmol and with a 99% radiochemical purity and 9% of *trans* isomer, L-(4.5-^{3}H)leucine 55 Ci/mmol were purchased from Radiochemical Centre, Amersham, England. Bovine albumin poor fatty acid was purchased from Miles Laboratories, Kankakee, Illinois.

Animals and Diets

Female Wistar rats Chbb Thom (Random)

*Members of the Carrera del Investigador Científico, Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina.

Germany, weighing 150-170 g, were used. The rats were fed the following diets ad libitum. Control diet: casein 25%, dextrine 66%, maize oil 5%, salt mixture 4% (6) and vitamins (7). Low protein diet: casein 5%, dextrine 88%, maize oil 5%, salt mixture 4% and vitamins.

The rats were divided into 2 groups after mating and were fed the diets indicated above throughout gestation. On the 20th day of gestation the mothers were decapitated and blood samples collected and livers removed.

The rats assigned to in vitro experiments were anesthetized using an intraperitoneal injection of sodium pentobarbital (50 mg/kg), and in each experiment 2 livers were perfused, one of them from a normal rat and the other from a protein deficient rat.

Perfusion Technique

The perfusion technique and the perfusion apparatus were the same as those described by Assimacopoulos et al. (8). The livers were perfused with 70 ml of Krebs-Henseleit buffer pH 7.4 containing 210 mg of glucose and 3 g of bovine serum albumin complexed with 150 μmoles of palmitic acid (9). After 20 min of perfusion, 1 ml of 0.15 M NaCl containing 0.7 μmoles of linoleic acid and 10 μCi of [1-^{14}C] linoleic acid-albumin complex was added, and the perfusion was continued for 120 min at a flow rate of 2.0 ml/min/g^{-1} tissue. In another trial [^{3}H] arachidonic acid was used instead of labeled linoleic acid. When [^{3}H] leucine incorporation into apoprotein was measured, 100 μCi of [^{3}H] leucine was added instead of the labeled fatty acids.

Isolation of VLDL

VLDL from serum and perfusate were isolated by ultracentrifugation at the medium density 1.007 according to the technique of Lindgren (10) in an MSE ultracentrifuge using a 43114 rotor at 40,000 rom for 18 hr at 10 C. The VLDL fraction was recentrifuged for purification, and its purity was checked by polyacrylamide gel electrophoresis (Lipofilm, Sebia, France).

Extraction and Analysis of Lipids

The lipids were extracted from an aliquot of the VLDL solutions, from the serum samples and from the livers by the method of Folch-Pi et al. (11).

The lipids were recovered from the original chloroform-methanol extracts and resolved into their components by thin layer chromatography (TLC) (12), and the amount of triacylglycerol was determined after this fraction was extracted from the silica using warm chloroform-methanol 2:1 v/v. The recovery from the silica was corrected by the use of authentic standard and quantified by glycerol analyses (13).

The incorporation of radioactivity into triacylglycerol from VLDL was measured after the samples were chromatographed, the silica scraped off from the plate, transesterified with methanolic HCl, extracted with light petroleum, evaporated and counted in a Packard Scintillation Counter.

Apo C Isolation and Quantification

Total proteins from serum VLDL were estimated by the modified Lowry assay (14). Apo C from VLDL was isolated by the method of Holmquist and Carlson (15), using acetone as organic solvent, and quantified by protein analysis. The efficiency of extraction and purity of the isolated apo C were checked by electrophoresis in 0.1% SDS on 10% polyacrylamide gel according to Weber and Osborne (16).

Isolation and Counting of Apolipoproteins of VLDL

The recentrifuged VLDL lipoproteins were dialyzed against distilled water pH 8.4 with NH_4OH and lyophilized. Delipidation was performed at 4 C on freeze-dried lipoproteins with ethanol/diethyl ether (17). Apolipoproteins were solubilized in 0.02 M sodium phosphate buffer pH 7.0, 1% in SDS and 1% in β-mercaptoethanol and placed in boiling water for 2 min. The apoproteins were then separated by electrophoresis as was previously described.

The radioactivity of individual apolipoproteins was determined in bands sliced from the stained gels according to Mahin and Lofberg (18). Identification of the different apoproteins was based on their different mobilities using the nomenclature of Swaney et al. (19), and by running samples of creatin phosphokinase (MW 40,000), trypsine (MW 24,000) and cytochrome (MW 11,700), Sigma Chemical Co., St. Louis, Missouri.

RESULTS

In Vivo Experiments

Protein depletion during pregnancy promotes a drastic increase in serum and liver triacylglycerol contents as is shown in Table 1. The amount of liver triacylglycerol and the serum triacylglycerol concentration of the protein depleted rats are double and triple, respectively, the values observed in the control animals.

The isolated VLDL particles show an increase in the triacylglycerol/apoprotein ratio promoted in the rat by protein depletion dur-

TABLE 1

Effect of Protein Depletion on the Amounts of Serum and Liver Triacylglycerol from Pregnant Rats

Experimental group	Serum triacylglycerol mg/100 ml	Liver triacylglycerol mg/g
Control (6)[a]	413.2 ± 102.1[b]	6.8 ± 2.3
Low protein (10)	1226.4 ± 414.5	13.8 ± 4.3
	P < 0.001	P < 0.01

[a]Numbers between parentheses indicate the number of animals in each group.
[b]Values are the mean ± SD.
Probability values are related to control group.

ing pregnancy, as can be seen in Table 2. Serum triacylglycerol is transported mainly as a core in the VLDL particles surrounded by a polar-lipid-protein environment.

In order to determine if the protein moiety of VLDL was defective in any particular apoprotein, the content of apo C was determined. The results show that apoprotein C is significantly reduced as component of the protein moiety of the VLDL isolated from protein depleted pregnant rats (Table 2).

In Vitro Experiments

It was suggested (20) that there is a circulating apoprotein which can be reused for VLDL formation without de novo synthesis, but if the liver is perfused with plasma free medium one can overlook the effect of the circulating apoprotein and even study the apoprotein synthesis of the secreted VLDL.

The per cent distribution of the [^{3}H]-leucine radioactivity incorporated into peptides of VLDL isolated from liver perfusates of control and protein deficient pregnant rats is shown in Figure 1. The radioactivity is distributed primarily among peptides bands located in 3 molecular weight regions of the SDS gels identified according to their electrophoretic mobilities as apo B, ARP and apo C (21). The significant decrease in the radioactivity found in the small C peptides from the protein depleted animals was coincident with a decrease in the amount of these rapidly migrating bands.

TABLE 2

Effect of Protein Depletion on the Triacylglycerol/Apoprotein Ratio and apo C Content of Apoproteins from Serum VLDL of Pregnant Rats

Experimental group	triacylglycerol / apoprotein	apoprotein % apo C
Control (4)[a]	8.2 ± 2.5[b]	35.0 ± 1.1
Low protein (5)	13.3 ± 2.8	24.5 ± 3.2
	P < 0.05	P < 0.001

[a]Number between parentheses indicates the number of animals.
[b]Values are the mean ± SD.
Probability values are related to control group.

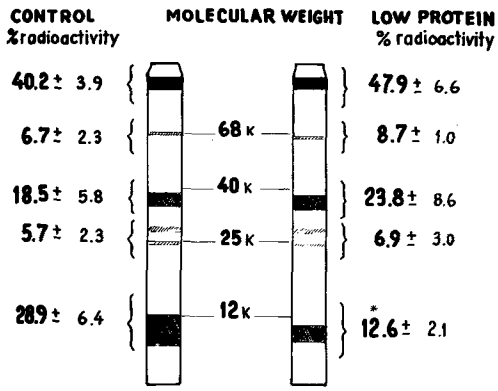

FIG. 1. Per cent distribution of [^{3}H] leucine radioactivity incorporated into apoproteins from VLDL isolated from liver perfusates of control and protein deficient pregnant rats. Data are the mean ± S.D. Each point represents the mean of three determinations.
*Significant difference from control.

The effect of protein depletion on the capability of the perfused liver to esterify polyunsaturated fatty acids into triacylglycerol also was measured. No significant differences were observed in the amount of radioactivity from labeled linoleic and arachidonic acids incorporated per μmol of VLDL traicylglycerol by the perfused livers from pregnant rats.

DISCUSSION

Pregnancy could affect lipid metabolism since hypertriglyceridemia in late gestation is observed in several animal species, including man and rat (22). Two possible mechanisms were

postulated to explain this elevated triacylglycerol concentration: first, an increased synthesis and second, an impaired removal of VLDL triacylglycerol (23). Published data favor triacylglycerol overproduction rather than underremoval as the mechanism of VLDL triacylglycerol accumulation in a well protein fed pregnant rat. However, when the protein intake was lowered as in the low protein diet group, an abnormal increase of serum triacylglycerol concentration was observed (Table 1). Coincident with this fact is the secretion by the liver of a defective VLDL particle which shows a relative decrease in their content of apoproteins as was suggested by the increase in the triacylglycerol apoprotein ratio.

Analysis of the apoprotein showed there was a decrease in the apo C content of VLDL, suggesting that its synthesis is indeed decreased in protein malnutrition. This assumption was confirmed by measuring the incorporation of [^{3}H] leucine into VLDL apoproteins by the perfused liver of protein depleted pregnant rats. Certainly [^{3}H] leucine incorporation into apo C peptides is drastically reduced in the secreted VLDL (Fig. 1).

On the other hand, protein deprivation did not affect polyunsaturated fatty acid incorporation into triacylglycerol of the newly secreted VLDL.

Taking together the results obtained from in vivo and in vitro experiments, let us deduce that a defective VLDL is secreted by the liver of protein depleted pregnant rats. The high triacylglycerol/protein ratio and the low content of apo C in these particles may influence lipoprotein metabolism throughout their effects on lipoprotein lipase (24). Thus, we can assume that the hypertriglyceridemia observed in the protein depleted rats during gestation may be caused by an impaired removal of VLDL triaclyglycerol (25) that could affect the normal supply of polyunsaturated fatty acids to the fetus.

ACKNOWLEDGMENTS

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REFERENCES

1. Crawford, M.A., Hassam, A.C., Williams, G., and Whitehouse, W.L. (1976) Lancet 1, 452-458.
2. Hassam, A.G., Sinclair, A.I., and Crawford, M.A. (1975) Lipids 1, 417-420.
3. Mercuri, O., De Tomás, M.E., and Itarte, H. (1979) Lipids 14, 822-825.
4. Pascaud, M., Rougier, A., and Delhaye, N. (1977) Nutr. Metab. 21, 310-316.
5. Hummel, L., Schwartze, A., Shirrmeister, W., and Wagner, H. (1976) Acta Biol. Med. Germ. 35, 1635-1641.
6. De Tomás, M.E., Mercuri, O., and Serres, C. (1983) J. Nutr. 113, 314-319.
7. Brenner, R.R., Mercuri, O., and De Tomás, M.E. (1962) J. Nutr. 77, 203-209.
8. Assimacopoulos-Jeannet, F., Exton, J.H., and Jeanrenaud, B. (1973) Am. J. Physiol. 225, 25-32.
9. Elovson, J. (1965) Biochim. Biophys. Acta 106, 480-494.
10. Lindgren, F.T. (1975) in Analysis of Lipids and Lipoproteins (Perkins, E.G., ed.) pp. 204-224, American Oil Chemists' Society, Champaign, Illinois.
11. Folch-Pi, J., Lees, M., and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497-509.
12. Malins, D.C., and Mangold, H.K. (1960) J. Am. Oil Chem. Soc. 37, 576-578.
13. Snyder, F., and Stephens, N. (1959) Biochim. Biophys. Acta 34, 244-245.
14. Kashyao, M.L., Hynd, B.A., and Robinson, K. (1980) J. Lipid Res. 21, 491-494.
15. Holmquist, L., and Carlson, K. (1977) Biochim. Biophys. Acta 493, 400-409.
16. Weber, K., and Osborne, M. (1969) J. Biol. Chem. 244, 4406-4412.
17. Glangeaud-Freudenthal, M.C., Burdin, J. Ayrault-Jarrier, M., and Polonouski, J. (1981) Biochimie 63, 485-494.
18. Mahin, T.D., and Lofberg, R.T. (1966) Analytical Biochem. 16, 500-509.
19. Swaney, B.J., Braithwaite, F., and Eder, A.H. (1977) Biochem. 16, 271-278.
20. Yagasaki, K., and Kametaka, M. (1978) Proc. Japan Acad. 54 (B) 464-468.
21. Marsh, J.B. (1976) J. Lipid Res. 17, 85-90.
22. Hummel, L., and Schirrmeister, W. (1974) Experientia 30, 637-641.
23. Hummel, L., Zimmerman, T., Schenk, H., Schwartz, A., Schirrmeister, W., and Wagner, H. (1978) Acta Biol. Med. Germ. 37, 259-266.
24. Shelburne, F., Hanks, J., Meyers, W., and Quarfordt, S. (1980) J. Clin. Inv. 65, 652-658.
25. Kashyap, M.L., Srivastava, S.L., Hynd, B.A., Perisutti, G., Brady, D.W., Gartside, P., and Glueck, C.J. (1978) Lipids 13, 933-942.

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Mass Spectrometric Evidence for the Presence of Platelet-Activating Factor (1-0-Alkyl-2-Acetyl-*sn*-Glycero-3-Phosphocholine) in Human Amniotic Fluid During Labor

JUN NISHIHIRA,[a] TERUO ISHIBASHI,*[,a] YOH IMAI[a] and TOSHIO MURAMATSU,[b]
[a]*Department of Biochemistry, Hokkaido University School of Medicine, Sapporo 060, Japan, and* [b]*Faculty of General Education, Tokyo Medical and Dental University, Ichikawa 272, Japan*

ABSTRACT

Evidence is presented for the existence of platelet-activating factor (PAF) in human amniotic fluid during labor by gas-liquid chromatographic (GLC) and mass-spectrometric (MS) analysis. The unique ether-linked phospholipid was identified only in an amniotic fluid obtained from women during labor, and its alkyl side chain was composed exclusively of octadecyl residue.
Lipids 19:907-910, 1984.

INTRODUCTION

Platelet-activating factor (PAF) was recognized initially in IgE-sensitized rabbit basophils by antigen stimulation (1,2). Its chemical structure, 1-alkyl, 2-acetyl and 3-choline moiety, is responsible for certain potent biological activities (3-5), including platelet aggregation, neutrophil activations, increase of vascular permeability, bronchoconstriction and the hypotensive effect (6,7). Recently we reported that the precise chemical nature of PAF is also essential for antigenic expression (8). The unique alkyl phospholipid subsequently was found by specific stimuli from various other cells such as platelets (9), neutrophils (10), peritoneal macrophages (11) and bone marrow-derived mast cells (12).

Billah and Johnston recently suggested the presence of PAF in human amniotic fluid by chromatographic mobility, platelet aggregation and chemical modifications (13). However, the very limited quantities of PAF in amniotic fluid make it difficult to obtain the final structural proof. The results of the present study using mass spectrographic analysis presented convincing evidence that PAF is contained in human amniotic fluid during labor. The structural determination showed that the chain length in 1-alkyl was exclusively 18:0. These findings deserve to be followed up in further studies on the physiological significance of PAF in labor.

EXPERIMENTAL

Materials

The following chemicals were obtained from commercial sources: Silicic acid (100 mesh) from Wako Chemical Co., pre-coated thin layer plate (LK6DF) from Whatman Co., N,O- bis (trimetylsilyl) trifluoroacetamide and trimethylchlorosilane from Tokyo Kasei Kogyo Co., phospholipase C (clostridium perfringens) from Sigma Chemical Co., Ovahormon (estradiol dipropionate) from Teikoku Zoki Co. 1-O-Alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (PAF), which was used as an authentic standard, was prepared semi-synthetically from ratfish (Hydrolagus colliei) liver oil as described previously (14). The alkyl moiety consisted of 16.7% hexadecyl and 78.2% octadecyl. All other chemicals were of reagent grade.

*To whom correspondence should be addressed.

Extraction and Isolation of PAF

The procedure described by Billah and Johnston (13) was used with the following modifications. One liter of fresh amniotic fluid containing a small amount of contaminated blood was collected during parturition from women having a normal pregnancy. The amniotic fluid was mixed with methanol (amniotic fluid/methanol = 2:3, v/v) for 1 hr at room temperature. After centrifugation of the mixture for 10 min at 10,000 × g, the resulting supernatant was mixed with 6 vol of chloroform and 1.4 vol of water. The lower phase was evaporated under nitrogen, and the total lipids, which were dissolved in a minimal volume of chloroform, were subjected to silicic acid column chromatography (15). After removing neutral lipids with chloroform, phospholipids were eluted with methanol. The methanol eluate was applied to thin layer chromatography (TLC) as described by Demopoulos et al. (16) using a solvent system of chloroform/methanol/water (65:35:6, v/v/v). The part corresponding to authentic PAF was

scraped from the plate and eluted with chloroform/methanol/water (1:2:0.8, v/v/v), then further purified, by rechromatography using a solvent system of chloroform/methanol/water (65:35:4, v/v/v). Extract from the silica was used as the purified PAF for characterization of the chemical structure and biological activities.

Gas-Liquid Chromatography-Mass Spectrometry

The purified PAF prepared as described above was hydrolyzed with phospholipase C (17) and then the acyl-ester was removed by mild alkaline hydrolysis (18). The dephosphorylated and de-acylated lipid product was converted to 1-O-alkyl-2,3-di-O-trimethylsilyl-glycerol at 70 C using N,O-bis (trimethylsilyl)-trifluoroacetamide, pyridine and trimethyl chlorosilane (6:3:1, v/v/v) (19,20) and applied to a GLC-MS (Joel, JMS-D300) equipped with a 1 m glass column containing 3% OV-1. Conditions for this column were as follows: injector, 250 C; column, 150-300 C at a rate of 8 C/min; ion source, 180 C; ionozing voltage, 30 eV.

Assay for Biological Activity of the Lipid Extract

Platelet aggregation was measured by the turbidimetric method using an aggregometer (21). PAF concentrations were calculated by comparing the aggregation obtained along with authentic PAF at known molarity. Measurements of uterine contractions were carried out according to the procedure described by Trautschold (22). For this experiment, female virgin rats were administered an intraperitoneal injection of Ovahormon (0.1 mg) 24 hr before hysterectomy.

RESULTS

Identification of 1-O-Octadecyl-2,3-Di-O-Trimethylsilyl-Glycerol

The sample prepared as described in "Experimental" was subjected to GLC and MS (Fig. 1). MS examination (Fig. 1B) of the one peak (retention time of 11 min) among 6 major peaks which appeared from the GLC (Fig. 1A) gave the following values m/z (percentage of relative intensity): M-15 (1.07), M-90 (1.27), M-180 (1.98), 205 (100), 133 (34.8), 130 (24), 117 (22.3), 75 (10.5), 71 (14.8) and 57 (15.9). The compound was identified as 1-O-octadecyl-2,3-di-O-trimethylsilyl-glycerol by comparison with a spectrum of the trimethylsilyl derivative of authentic PAF (20). Although the other 5 peaks in Figure 1A also were examined by MS analysis, it was not possible to identify them as known substances, such as sphingomyelin or lysolecithin, which are possible contaminants because of their

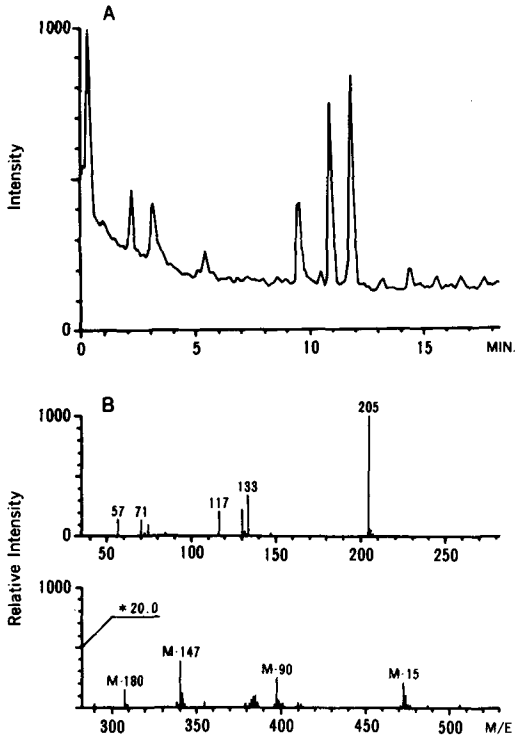

FIG. 1. Gas-liquid chromatography (A) and mass spectrometric (B) analysis of the sample extracted. The sample preparation and column conditions are described in "Experimental."

similar chromatographic behavior to PAF. On the contrary, no PAF derivative was detected in the amniotic fluid obtained by transuterine amniocentesis from full term pregnant women who showed hyperamniosis but no signs of labor (data not shown). Ten samples in each group were analyzed.

Biological Activities of the Isolated PAF

The sample extract induced a dose-dependent aggregation of washed rabbit platelets (data not shown). Furthermore, the sample provoked a significant muscle contraction of rat uterus (Fig. 2). The intensity of muscle contraction was dependent on the amount of extract added. However, more detailed quantification of PAF concentration was not performed. On the other hand, the PAF activities in amniotic fluid before the onset of labor could not be determined by these biological assays, or by the MS analysis described above.

DISCUSSION

In the present studies definite evidence was obtained for the presence of PAF in human

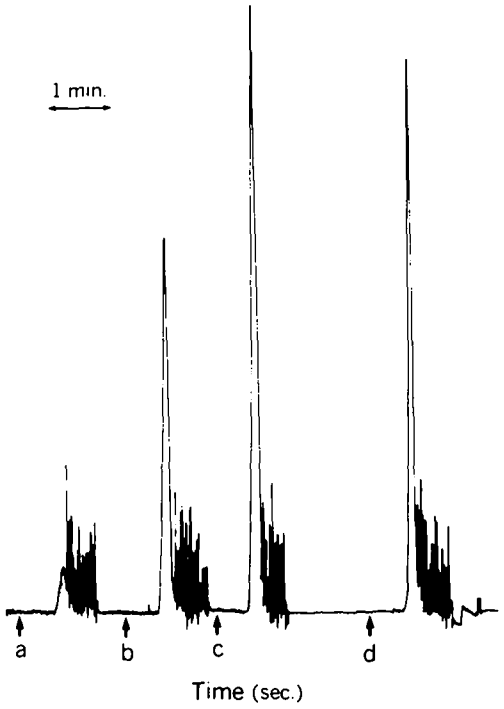

FIG. 2. Kymographic tracing of the contracting responses of rat uterine muscle by the sample extracted or the authentic PAF standard used. The muscle strip was exposed to 500 pM (a), 750 pM (b) and 1 nM (c) of the authentic PAF, respectively, and to 1/20 (d) of the sample extracted from 1 liter of amniotic fluid. Arrows indicate the times of an addition of PAF, which provoked the muscle contraction within 28 sec after initiation.

amniotic fluid during labor by GC and MS analysis (Fig. 1). This finding provides a strong impetus for future studies dealing with the cellular origin and molecular mechanisms of PAF secretion during labor. With regard to the origin of PAF in amniotic fluid, it is considered most likely to be released from the fetus, since amniotic fluid is composed primarily of fetal urine and lung secretions. Billah and Johnston have found PAF in fetal urine and in the pellet fraction of amniotic fluid, which is rich in lamellar bodies secreted by the fetal lung (13). Concerning fetal urine, the alkyl side chains of antihypertensive polar renomedullary lipid (APRL) isolated from the kidney, the structure of which is known to be the same as PAF (24), are composed primarily of hexadecyl (67%), with octadecyl (4%) appearing only in residues.

This composition of PAF is remarkably different from that presented here, which indicates the exclusive presence of octadecyl (Fig. 1). This molecular distinction of the side chain may deny the hypothesis of the renal origin of PAF in amniotic fluid. On the other hand, Prevost et al. recently reported that PAF was released in pulmonary alveolar fluid as a consequence of hypoxia (23). These observations seem to be further implications of the fetal lung origin of PAF in amniotic fluid.

Several lines of evidence support the idea that prostaglandins participate in the initiation of uterine contractions in human labor (25,26). Although the physiological function of PAF in amniotic fluid remains to be clarified, this communication has shown that PAF indeed induced significant uterine contractions (Fig. 2). PAF may work in conjunction with other lipid mediators such as leukotriens and thromboxane A_2 (27-30). In order to obtain further insights into the biochemical mechanism(s) involved in the initiation of human parturition, the roles of PAF during labor need to be elucidated and defined in greater detail.

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REFERENCES

1. Henson, P.M. (1976) J. Exp. Med. 143, 937-952.
2. Henson, P.M. (1977) J. Clin. Invest. 60, 481-490.
3. Hanahan, D.J., Munder, P.G., Satouchi, K., McManus, L., and Pinckard, R.N. (1981) Biochem. Biophys. Res. Commun. 99, 183-188.
4. Satouchi, K., Pinckard, R.N., McManus, L.M., and Hanahan, D.J. (1981) J. Biol. Chem. 256, 4425-4433.
5. Wykle, R.L., Miller, C.H., Lewis, J.C., Schmitt, J.D., Smith, J.A., Surles, J.R., Piantadosi, C., and O'Flaherty, J.T. (1981) Biochem. Biophys. Res. Commun. 100, 1651-1658.
6. Pinckard, R.N., McManus, L.M., and Hanahan, D.J. (1982) in Advances in Inflammation Research (Weissman, G., ed.) Vol. 4, pp. 147-180, Raven Press, New York.
7. Muirhead, E.E., Byers, L.W., Desiderio, D.M., Brooks, B., and Brosius, W.M. (1981) Fed. Proc. 40, 2285-2290.
8. Nishihira, J., Ishibashi, T., and Imai, Y. (1984) J. Biochem. 95, 1247-1251.
9. Chignard, M., Le Couedie, J.P., Tence, M., Vargaftig, B.B., and Benveniste, J. (1979) Nature 279, 799-800.
10. Clark, P.O., Hanahan, D.J., and Pinckard, R.N. (1980) Biochim. Biophys. Acta 628, 69-75.
11. Mencia-Huerta, J.M., and Benveniste, J. (1979) Eur. J. Immunol. 9, 409-415.
12. Mencia-Huerta, J.M., Lewis, R.A., Razin, E., and Austen, K.F. (1983) J. Immunol. 131, 2958-2964.
13. Billah, M.M., and Johnston, J.M. (1983) Biochem. Biophys. Res. Commun. 113, 51-58.
14. Muramatsu, T., Totani, N., and Mangold, H.K. (1981) Chem. Phys. Lipids 29, 121-127.

15. Rodwell, M., Scow, R.O., and Chernick, S.S. (1964) J. Biol. Chem. 239, 385-391.
16. Demopoulos, C.A., Pinckard, R.N., and Hanahan, D.J. (1979) J. Biol. Chem. 254, 9355-9358.
17. Satouchi, K., Pinckard, R.N., McManus, L.M., and Hanahan, D.J. (1981) J. Biol. Chem. 256, 4425-4432.
18. Blank, M.L., Snyder, F., Byers, L.W., Brooks, B., and Muirhead, E.E. (1979) Biochem. Biophys. Res. Commun. 90, 1194-1200.
19. Myher, J.J., and Kuksis, A. (1974) Lipids 9, 382-390.
20. Myher, J.J., Marai, L., and Kuksis, A. (1974) J. Lipid Res. 15, 586-592.
21. Born, G.V.R. (1962) Nature 194, 927-929.
22. Trautschold, I. (1970) in Handbook of Experimental Pharmacology (Erdös, E.G., and Wilda, A.F., eds.) Vol. 25, pp. 52-81, Springer-Verlag, Berlin.
23. Prevost, M.L., Cariven, C., Simon, M.F., Chap, H., and Blazy, L.D. (1984) Biochem. Biophys. Res. Commun. 119, 58-63.
24. Smith, K.A., Prewitt, R.L., Byres, L.W., and Muirhead, E.E. (1981) Hypertension 3, 460-470.
25. Wiquist, N., and Wilhelmsson, L. (1979) Gynecol. Obstet. Invest. 10, 1-8.
26. Satoh, K., Yasumizu, T., Fukuoka, H., Kinoshita, K., Kaneko, Y., Tsuchiya, M., and Sakamoto, S. (1979) Am. J. Obstet. Gynecol. 133, 886-890.
27. Okita, J.R., McDonald, P.C., and Johnston, J.M. (1982) J. Biol. Chem. 257, 14029-14034.
28. Chilton, F.H., O'Flaherty, J.T., Walsh, C.E., Thomas, M.J., Wykle, R.L., DeChatelet, L.R., and Waite, B.M. (1982) J. Biol. Chem. 257, 5402-5407.
29. Chilton, F.H., O'Flaherty, J.T., Ellis, J.M., Swendsen, C.L., and Wykle, R.L. (1983) J. Biol. Chem. 258, 7268-7271.
30. Hopkins, N.K., Lin, A.H., and Gorman, R.R. (1983) Biochim. Biophys. Acta 763, 276-283.

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Effect of Dietary Vitamin E Upon Fluorescent Compounds of the Rat Uterus

A. SAARI CSALLANY*, K. LUND AYAZ and **B. ZASPEL MENKEN,** *Department of Food Science and Nutrition, University of Minnesota, 1334 Eckles Avenue, St. Paul, MN 55108*

ABSTRACT

Several water soluble and organic solvent soluble fluorescent peaks were observed in extracts of uteri from vitamin E deficient or supplemented rats following Sephadex column chromatography and spectrophotofluorometric analysis. Levels of one of the organic and two of the water soluble fluorescent peaks were found to be significantly higher in the uteri of vitamin E deficient rats than in vitamin E supplemented rats. Some of these fluorescent fractions may contribute to the brown discoloration that is known to occur in the uterus of vitamin E deficient animals.
Lipids 19:911-915, 1984.

INTRODUCTION

It has been established that a brown discoloration of the uterus occurs when rats become vitamin E deficient (1), resulting in an increase in fluorescence in the uterus (2). This fluorescence is thought to arise from peroxidation products of the lipid components of cellular material, sometimes referred to as ceroid-lipofuscin pigments (3). These pigments have been postulated to play a role in the fetal resorption process (4) and irreversible sterility (5) seen in vitamin E deficient rats. Desai et al. (6) have reported more fluorescence in the water soluble portion of the uterus in the vitamin E deficient rat than in the organic portion of the extract. The present study was undertaken to examine the fluorescent characteristics of organic and water soluble extracts obtained from the uteri of rats that have been fed a vitamin E deficient or supplemented diet for a one-year period.

MATERIALS AND METHODS

Animals and Diets

Twenty female, weanling Sprague-Dawley rats (Sprague-Dawley, Madison, Wisconsin) were fed either a basal, vitamin E deficient diet or a basal diet supplemented with d-α-tocopheryl acetate (300 mg/kg diet, Sigma Chemical Company, St. Louis, Missouri). The diet, containing 8% vacuum distilled corn oil, was identical to that described previously (7) except that it contained no added DL-methionine. Diet and water were provided ad libitum from weaning to one year of age, at which time the rats were killed by decapitation. The uteri were removed and immediately frozen at -70 C until extraction.

*To whom correspondence should be addressed.

Extraction of Uteri

The uterus from each animal was accurately weighed, homogenized and extracted in chloroform:methanol (2:1, v/v) and washed with water as described by the method of Csallany and Ayaz (8). The organic (chloroform) layer was dried over anhydrous sodium sulfate, and the solvent was removed over a rotary evaporator. The water-methanol layer was filtered to remove any insoluble material and lyophilized using a Virtis freeze drying apparatus.

Column Chromatography of Uteri Extracts

The organic layer of the tissue extract was chromatographed on a Sephadex LH-20 column in chloroform:methanol (1:9, v/v) as previously described (8). The dried water extract was brought up to a volume of 0.1-1.0 ml with water, and a known aliquot (100-250 μl) was applied to a 1.5 × 35 cm Sephadex G-25 (medium particule size) column and eluted with 0.02% sodium azide. Two-ml fractions were collected from both the Sephadex LH-20 and G-25 columns, and fluorescent measurements were made of each.

Quantitative Fluorescence Measurements

Fluorescent excitation and emission spectra were determined for each fraction eluting from the Sephadex column using an Aminco-Bowman Ratio Spectrophotofluorometer (American Instrument Co., Silver Spring, Maryland) as described previously (9). A Hanovia Xenon Lamp was used for the excitation light source. From the spectra, maximum excitation and emission wavelengths were determined for each eluting peak collected from the Sephadex LH-20 or Sephadex G-25 columns. Every fraction was read at these predetermined wavelengths, but each peak had its own excitation and emission

maxima. The instrument was standardized daily to read 100 relative fluorescent units against a quinine sulfate solution (1 μg/ml 0.1 N H_2SO_4) at an excitation wavelength of 350 nm and an emission wavelength of 435 nm.

Statistical Methods

The total fluorescence of each peak eluted from the column was measured and calculated as relative fluorescence units per gram of wet tissue. Statistical comparisions of the mean fluorescence values of each peak were analyzed using the two-tailed Student's t distribution (10) to determine significant differences between the vitamin E deficient and vitamin E supplemented groups.

RESULTS

Animals

Growth curves were used to assess the vitamin E status of the rats. After 17 weeks on the vitamin E deficient diet, the rats began to show noticeable decreases in the rate of weight gain compared to vitamin E supplemented rats. This weight difference continued to increase and by one year of age the vitamin E deficient rats weighed 25% less than the vitamin E supplemented rats.

Column Chromatography

Elution profiles for organic and water soluble extracts from vitamin E supplemented rat uteri were qualitatively identical to those of the vitamin E deficient rats. A typical elution profile for the organic uterus extract from a vitamin E deficient rat is shown in Figure 1. The first peak eluting from the Sephadex LH-20 column (fractions #7-17) had maximum excitation and emission wavelengths (ex/em) at 350 nm and 435 nm, respectively. This has been observed previously in other tissues (7), and the material has been referred to as the "organic solvent soluble lipofuscin pigment." The second and third peaks (fractions #18-40), both exciting and emitting maximally at 240 nm/350 nm, are not well separated by Sephadex LH-20 column chromatography. Retinol (350 nm/495 nm) was not detected in the organic portion of the uteri extracts as observed previously in other tissues such as the liver (8).

A typical Sephadex G-25 elution profile for the water-soluble uterus extract is shown in Figure 2. Four fluorescent peaks were observed with maximal excitation and emission wavelengths as follows (in order of elution): peak 1 (fractions #10-16), 275 nm/350 nm; peak 2 (fractions #23-37), 270 nm/310 nm; and peaks 3 and 4 (fractions #23-46), 275 nm/350 nm. Peaks with the same fluorescence characteristics and elution behavior have been observed in the rat liver (Csallany, A.S., and Menken, B. Zaspel, unpublished data) and in the mouse liver (9). Peak 3 in Figure 2, however, has not been observed in the mouse liver.

When the same water extract was chromatographed on a 1.5 x 35 cm Sephadex G-15 column in 0.02% sodium azide, one additional peak (peak 2) was found with maximal excitation and emission wavelengths of 275 nm/350 nm (Fig. 3). Although separation of the fluorescent compounds was improved, the Sephadex G-25 column was used routinely for the separation of the water extracts because Sephadex G-15 column chromatography took 7-8 hr longer.

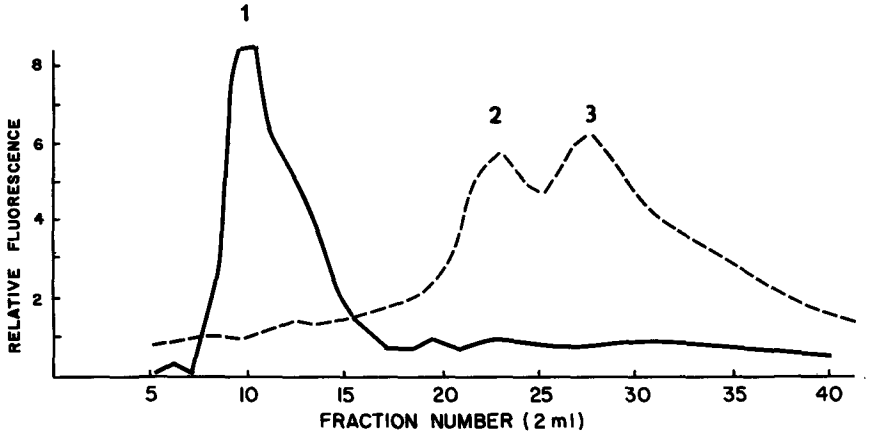

FIG. 1. Organic soluble fluorescent peaks of rat uterus eluted from a Sephadex LH-20 column with chloroform:methanol (1:9, v/v). Excitation wavelength/emission wavelength 350 nm/435 nm (——) and 240 nm/350 nm (- - - -).

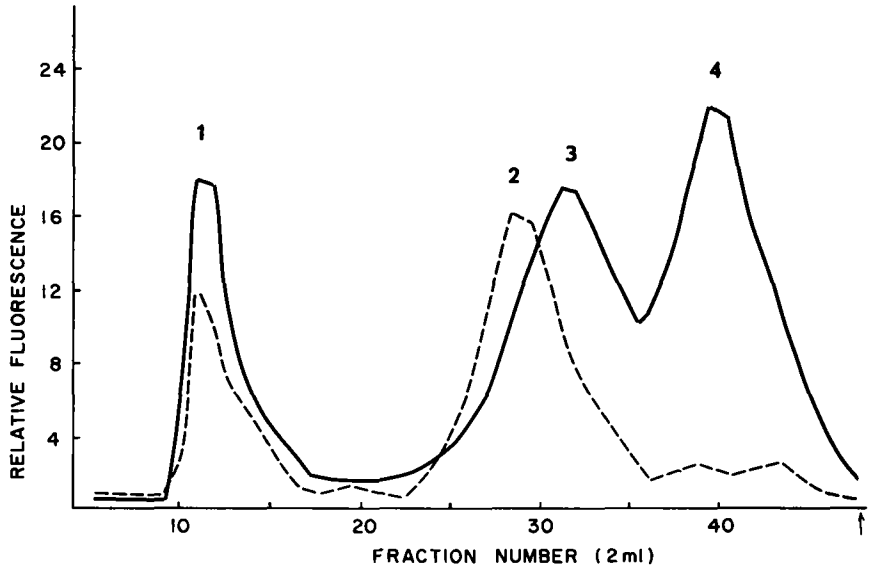

FIG. 2. Water soluble fluorescent compounds of rat uterus eluted from a Sephadex G-25 medium particle size column with 0.02% sodium azide. Excitation wavelength/emission wavelength: 275 nm/350 nm (——) and 270 nm/350 nm (- - - -).

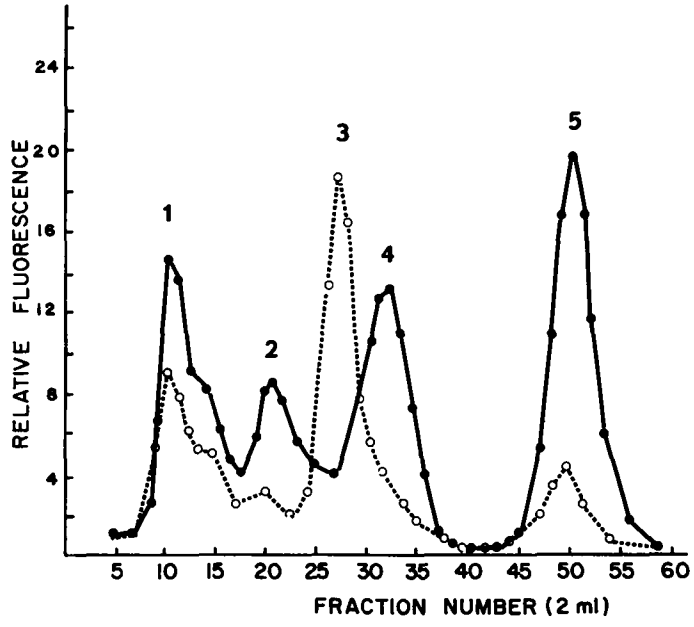

FIG. 3. Water-soluble fluorescent compounds of rat uterus eluted from a Sephadex G-15 column with 0.02% sodium azide. Excitation wavelength/emission wavelength 275 nm/350 nm (——) and 270 nm/310 nm (- - - -).

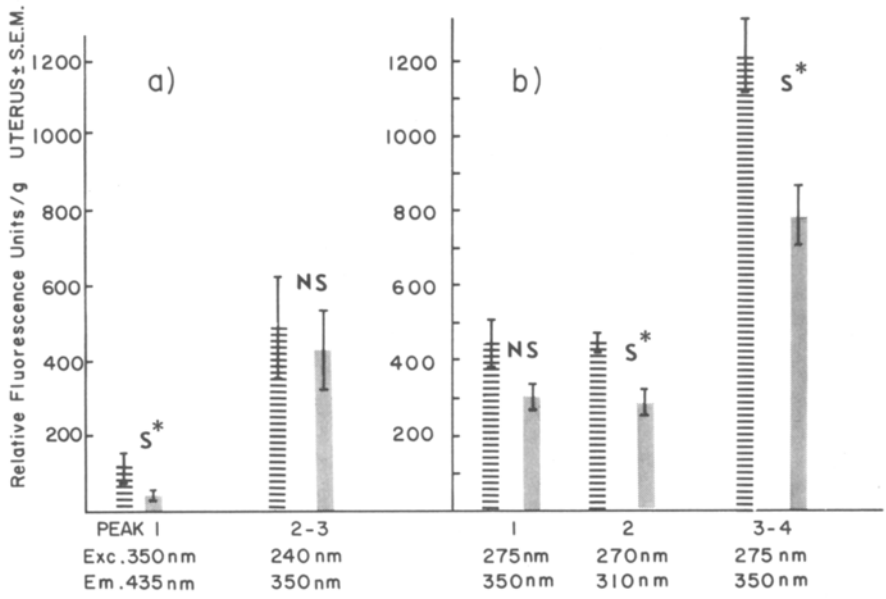

FIG. 4. Summary of the fluorescent compounds from the (a) organic and (b) aqueous extracts of vitamin E deficient (≡) and vitamin E supplemented (⁘) rat uteri. Each bar represents the mean ± S.E.M. for 6 animals. NS = not significant. S* = significant (Student's t test, $p < 0.05$).

Analysis of Uteri from Vitamin E Deficient and Supplemented Rats

Visual examination of the uteri from the vitamin E deficient rats revealed a uniform, dark brown pigmentation whereas the vitamin E supplemented uteri had a light pink color. This characteristic in vitamin E deficiency has been well documented (1,2,4-6).

The results from the fluorescence analysis of the uteri from the vitamin E deficient and vitamin E supplemented rats are shown in Figure 4. In the organic phase of the extracts the 350 nm/435 nm compound, the "organic solvent soluble lipofuscin pigment," was significantly higher in concentration in the vitamin E deficient group than the vitamin E supplemented group. We previously have reported similar increases in this compound in the liver but not in other tissues of vitamin E deficient mice (7,11). The second group of organic solvent soluble compounds (a combination of peaks 2 and 3 of Fig. 1) with maximum excitation and emission wavelengths of 240 nm/350 nm was not significantly different for the deficient and supplemented rats.

The fluorescent compounds from the water-methanol phase of the uteri extracts are shown in Figure 4b. The first peak with ex/em maxima at 275/350 nm showed no significant difference between the vitamin E deficient and supplemented groups. The second fluorescent compound (270 nm/310 nm) was significantly higher in the vitamin E deficient animals. The third fluorescent compound with ex/em maxima of 275 nm/350 nm (calculated as a combination of peaks 3 and 4) also was significantly higher in vitamin E deficient animals.

DISCUSSION

The irreversible brown pigmentation of the rat uterus that occurs in dietary vitamin E deficiency is commonly thought to be due to increased accumulation of fluorescent, ceroid-lipofuscin type pigments (1-5). In the present study an attempt was made to further separate and determine the fluorescent materials in the uterus under various dietary conditions. Present data show that the 350 nm/435 nm peak in the organic phase and the 270 nm/310 nm and the later eluting 275 nm/350 nm peaks in the aqueous phase were higher in concentration due to vitamin E deficiency. About 3 times as much fluorescence was found in the water-soluble portion of the uteri extracts as in the organic portion. The fluorescence of the organic and water-soluble extracts of deficient rat uteri, however, does not seem to account for the heavy brown discoloration observed. The same fluorescent peaks were found in vitamin E deficient and supplemented rat uteri, but the differences

in fluorescence between the significant dietary groups were twice or less. It appears that additional fluorescence and possibly the major portion of the fluorescence in brown uteri must be present in the insoluble part of the tissue. It has been reported by our laboratory that the washed, insoluble interphase material from 2:1 chloroform:methanol liver extracts has a total fluorescence much greater than that of the water extract (12) at 280 nm/340 nm. This is not surprising as a number of proteins, especially the larger ones, would be expected to precipitate out in the presence of chloroform and methanol and remain insoluble as denatured proteins. The results of this study appear to indicate that some of the fluorescent fractions observed may contribute to increased fluorescence known to occur in the "brown uterus" of vitamin E deficient rats; however, the insoluble interphase may contain more of the fluorescent compounds responsible for this severe pigmentation.

ACKNOWLEDGMENTS

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REFERENCES

1. Martin, A.J.P., and Moore, T. (1936) Chem. and Ind. 55, 236.
2. Moore, T., and Wang, Y.L. (1947) Br. J. Nutr. 1, 53-64.
3. Porta, E.A., and Hartroft, W.S. (1969) in Pigments in Pathology (Holman, M., ed.) p. 191, Academic Press, New York, NY.
4. Horowitz, I., and Hartroft, W.S. (1971) J. Nutr. 101, 959-966.
5. Raychaudhuri, C., and Desai, I.D. (1971) Science 173, 1028-1029.
6. Desai, I.D., Fletcher, B.L., and Tappel, A.L. (1975) Lipids 10, 307-309.
7. Csallany, A.S., Ayaz, K.L., and Su, L.-C. (1977) J. Nutr. 107, 1792-1799.
8. Csallany, A.S., and Ayaz, K.L. (1976) Lipids 11, 412-417.
9. Manwaring, J.D., and Csallany, A.S. (1981) J. Nutr. 111, 2172-2179.
10. Snedecor, G.W., and Cochran, W.G. (1967) in Statistical Methods (Snedecor, G.W., and Cochran, W.G., eds.) pp. 59-60, Iowa State University Press, Ames, IA.
11. Csallany, A.S., and Ayaz, K.L. (1978) Arch. Environ. Health 33, 285-291.
12. Manwaring, J.D., Csallany, A.S., and Zaspel, B.J. (1981) Fed. Proc. 40, 894.

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Cholesteryl Ester Hydrolysis in Rat Liver Cytosol. Modulation by Female Sex Hormones

J.M. GANDARIAS, M. LACORT and **B. OCHOA***, *Department of Physiology and Biochemistry, Faculty of Medicine, University of Pais Vasco, Bilbao Ap. 699, Spain*

ABSTRACT

The regulation of cholesterol ester hydrolase activity by female sex hormones was studied in cytosolic preparations from female rat liver. The investigation was undertaken in order to determine whether a reduction in the enzyme activity might be responsible for the increased content of esterified cholesterol found in rat liver after estradiol or progesterone treatments. The single injection of estradiol (0.75mg/100 g) or progesterone (1.50mg/100g) produced respectively significant decreases and increases in sterol hydrolase activity. Both opposite effects were noted after a similar lag period of 3-4 hr and were of short duration. No alterations were observed in rats receiving short-term treatments. When hormones were added to the incubation medium, the activity of cholesterol ester hydrolase decreased progressively with increasing concentrations of hormones. Kinetic studies demonstrate that both estradiol and progesterone compete with the substrate (cholesteryl oleate) for the active center. The findings of the present paper exclude a direct relationship between hepatic hydrolytic activity and lipid deposition. However, they provide evidences that female sex hormones act as modulatory agents of the hydrolysis of cholesteryl esters in rat liver cytosol and suggest that other factors besides competitive inhibition are involved in such regulatory effects.
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INTRODUCTION

Hepatic cholesteryl esters, which derive primarily from the uptake and degradation of plasma lipoproteins, are hydrolyzed in the liver cells through the action of the enzyme cholesterol ester hydrolase (also called cholesterol esterase, EC 3.1.1.13). The resulting free cholesterol as well as the endogenous cholesterol can be catabolized, reesterified and stored or secreted from the liver. Free and esterified cholesterol are then subject to constant turnover, and the enzyme cholesterol esterase plays an important role in controlling the supply of free sterol from its storage form. The existence of at least 3 cholesterol ester hydrolytic activities in different hepatic subcellular locations to which specific biological functions have been attributed has been described (1,2). The cytosolic enzyme, which acts at a pH near neutrality, has been implicated mainly in the breakdown of plasma lipoproteins internalized in the liver by endocytic processes (3,4).

It has been postulated that the hepatic esterified cholesterol content reflects net plasma cholesterol uptake by the hepatocyte (5); so, an increased level of this lipid in the liver could indicate some alteration in the rate of this process. Previous results in our laboratory showed that in estradiol and progesterone treated rats, hepatic esterified cholesterol is increased drastically, while the initial rate of plasma cholesterol esterification was not altered and, therefore, the turnover and hepatic catabolism of high and low density lipoproteins were not supposed to be substantially changed (6,7). However, there are experimental evidences that estrogens induce specific cell surface receptors for apoB and apoE containing lipoproteins (8-11) including remnants of chylomicrons and very low density lipoproteins which are quantitatively the most important particles in the supply of cholesteryl esters to the liver (12,13). Thus, we found it interesting to establish the relative influence of female sex hormones on hepatic processes related to the uptake and degradation of lipoproteins.

In the present study we have determined the cholesterol ester hydrolase activity from rat liver cytosol under endocrine conditions in which enhancement in hepatic esterified cholesterol was induced and also after a single injection of hormones. Because we have observed significant modifications in the enzyme activity after the administration of a single dose of estradiol or progesterone, we also have investigated the influence of the presence of these hormones on cholesterol ester hydrolase activity and on its kinetic parameters.

MATERIALS AND METHODS

Estradiol, progesterone, oleic acid, bovine serum albumin and unlabeled cholesteryl oleate were obtained from Sigma Chemical Co., St. Louis, Missouri. Cholesteryl [1-^{14}C]oleate (sp. act. 50 Ci/mol) was purchased from the Radio-

*To whom correspondence should be addressed.

chemical Center, Amersham, United Kingdom) and was used without further purification after being checked by thin layer chromatography. Naphtalene, 1-4dioxane, 2,5-diphenyloxazole (PPO) and 1,4-di-2,5-phenyloxazolilbenzene (POPOP), were from Hopkin & Williams Ltd, Chadwell Heat, Essex, United Kingdom. All other chemicals used were reagent grade.

Female Wistar rats (160-210g) were maintained on standard laboratory chow and tap water "ad libitum" and were exposed to an alternating light cycle of 12 hr (light from 7:00 a.m.). When the effects of short-term treatments of pharmacological doses were studied, groups of 4 rats were given daily injections of estradiol (1.50mg/100g) or progesterone (2.5 mg/100g) dispersed in 0.5ml, 1,2-propylenglycol (10% in saline solution) for 3 consecutive days. Control animals received isovolumetric amounts of hormone vehicle. Animals had free access to food until they were killed by decapitation 24 hr after the last dose of hormone. In the studies for the effects of a single injection of hormones, rats were given estradiol (0.75mg/100g) or progesterone (1.50mg/100g) and were killed at intervals varying between 1 and 24 hr after the treatment. All experiments began at 8:00 a.m.

The blood from control and treated animals was collected into heparinized tubes and the plasma was removed for hormone determinations. Plasma estradiol and progesterone levels were measured with a commercially available radioimmunoassay (CEA SORIN, RIA Kit).

Cholesterol ester hydrolase assay: The liver was excised from freshly killed rats, rinsed in cold saline, weighed and homogenized in a Potter-Elvehjem homogenizer with 9 vol. of 35 mM sodium phosphate buffer (pH 7.45). The homogenates were centrifuged at 2,000xg for 30 min, and the resulting supernatant was recentrifuged at 105,000xg for 60 min to obtain the particle-free supernatant (cytosol fraction). Care was taken to remove the floating lipid layer. Defatted cytosol preparations were used habitually as the enzyme source. Cholesterol ester hydrolase activity was measured in terms of the release of [1-^{14}C] oleic acid from the cholesteryl [1-^{14}C] oleate substrate according to the method of Traynor and Kunze (14). A model incubation mixture contained, in a total volume of 2 ml, 100 nmol of cholesteryl [1-^{14}C] oleate (sp. act. 0.22 Ci/mol) dispersed in 50 μl acetone, 750 μl of enzyme source and 35 mM sodium phosphate buffer (pH 7.45). The assay was run for 45 min at 37 C in a metabolic shaker. When estradiol or progesterone was added to the incubation medium, they were dissolved in 10 μl acetone and injected with a Hamilton syringe. 10 μl acetone were added to the control incubates. In all experiments blanks containing buffer in place of the enzyme protein were done and averaged 1% of the total substrate radioactivity. The reaction was stopped and the lipids were extracted as described by Nilsson (3). The lipidic extract was separated by TLC, and radioactive areas were scraped and suspended in 10 ml of a dioxan-based scintillation cocktail. Radioactivity was measured in a Searle Mark II liquid scintillation counter. The percentage of cholesteryl esters hydrolyzed was calculated according to Nilsson's equation (3), and the unit of enzyme was arbitrarily defined as the amount of enzyme which hydrolyzes 1 pmol of substrate per hr at 37 C. Protein was determined by the method of Lowry et al. (15) using bovine serum albumin as standard.

Liver lipids were extracted, separated and cholesteryl esters determined as described earlier (6).

The kinetic analysis of the results was performed with a Superbrain INTERTEC microcomputer using a weighted least squares fit of the data points to a double reciprocal plot (Lineweaver-Burk representation). Statistical significance was determined by the Student's t-test.

RESULTS

The optimal conditions for measuring the cytosolic cholesterol ester hydrolase activity were chosen after a previous set of experiments was performed. The hydrolase activity proved to be linear with time up to 60 min and with protein concentration up to 8 mg/ml, when the substrate was present at a 50 μM concentration. The effect of increasing substrate concentrations and the Lineweaver-Burk representation also were done. A typical Michaelis & Menten kinetic was found (Fig. 1).

The effects of short-term treatments of pharmacological doses of estradiol and progesterone on cytosolic cholesterol ester hydrolase activity are reflected in Table 1. Data show that the administration of both hormones over 3 consecutive days resulted in no significant changes in hydrolase activity. It should be pointed out that animals receiving hormonal injections had plasma levels of estradiol of more than 700 pg/ml (control, 7.2 pg/ml) and of progesterone of more than 500 ng/ml (control, 14.4 ng/ml) at the time of killing (Table 1).

The time-response studies of cholesterol esterase activity and of hepatic cholesteryl ester content in rats given a single dose of the hormone are summarized in Figure 2. Estradiol

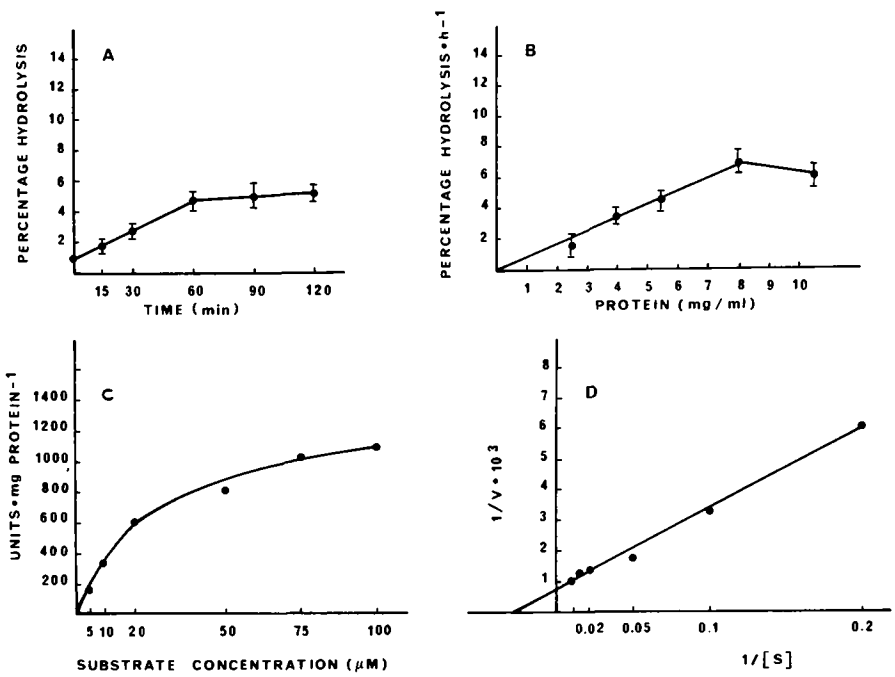

FIG. 1. Cholesterol ester hydrolase activity from rat liver cytosol. A - Time-course reaction. The protein in the incubate was 5.8 mg/ml. B - Dependence of the rate of hydrolysis with protein concentration. The incubation ran for 45 min. C - Dependence of the rate of hydrolysis with substrate concentration. The protein in the incubate was 3 mg/ml. D - Double-reciprocal design of the values given in C. The enzyme activity is expressed as the percentage of the total substrate hydrolyzed (A), as the percentage of the substrate hydrolyzed per hour (B) and as units per mg protein (C,D). Each point is the mean of 4 determinations. Bars represent the S.E. of the mean.

TABLE 1

Effect of Estradiol (1.50mg/100g) and Progesterone (2.50mg/100g) Administration for 3 Days on Cytosolic Cholesterol Ester Hydrolase Activity from Rat Liver and on Plasma Estradiol and Progesterone Levels

Group	Cholesterol esterase (units/mg protein) (4)	Hormone concentration (pg/ml plasma) (8)	
		Estradiol	Progesterone
Control	652 ± 44	7 ± 3	—
Estradiol-treated	617 ± 37	798 ± 87*	—
Control	557 ± 32	—	14,430 ± 1,056
Progesterone-treated	567 ± 27	—	558,640 ± 32,572*

Results are expressed as mean ± S.E.
The number of observations are given in parenthesis.
*Significantly different from control. $P \leq 0.005$.

caused a significant decrease in the enzyme activity 3 hr after the treatment (about 50%); however, the progesterone increased the cholesterol ester hydrolase activity, this effect being maximal 4-6 hr after the injection. Both opposite effects diminished gradually until the enzyme reached normal values at 24 hr. The lack of response observed following 24 hr of the injection agrees with the results obtained in rats treated for 3 days in which the enzyme also was measured 24 hr after the last dose. After 4 hr of hormone treatments, the amount of hepatic

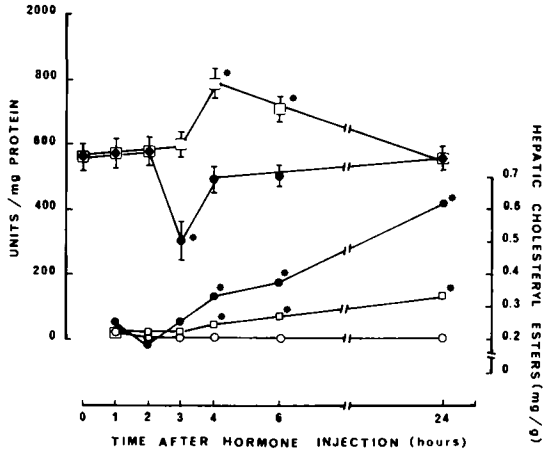

FIG. 2. Time-response of cytosolic cholesterol ester hydrolase activity and liver esterified cholesterol following administration of a single dose of (•) estradiol (0.75mg/100g), (□) progesterone (1.5mg/100g) or (○) vehicle. The studies were performed using 3 rats per group. Enzyme activity was determined in pooled cytosolic fractions from rats killed at the indicated times following the hormone injection *8:00 a.m.); rats from control group were sacrificed at 9:00 and 11:00 a.m. and noon to average some diurnal fluctations. Esterase activity is expressed as units/mg protein and hepatic cholesteryl esters level as mg/g wet weight. The points are the mean of triplicate determinations. Bars represent the S.E. of the mean.

*Significantly different from control. $P \leq 0.005$.

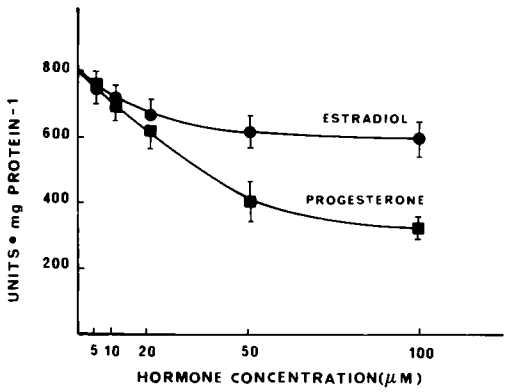

FIG. 3. In vitro effect of estradiol and progesterone on cholesterol ester hydrolase activity. Rat liver cytosol preparations were incubated with varying concentrations of estradiol and progesterone between 5 and 100 μmol/l. Protein concentration in the incubates was 2.8 mg/ml. The points are the average of triplicate determinations of a selected experiment. Bars represent the S.E. of the mean.

*Significantly different from control.

cholesteryl esters rose significantly, reaching the maximum level at 24 hr.

To test the direct effect of estradiol and progesterone on cytosolic cholesterol esterase, incubations were carried out in the presence of various hormone concentrations ranging from 5 to 100 μM. Data depicted in Figure 3 show that enzyme activity decreased gradually with increasing amounts of the hormones and that inhibition was significant at concentrations higher than 10 μM. Additional experiments were designed to verify the existence of interactions between the steroid molecule and the substrate of the reaction. The dependence of the hydrolytic activity with enzyme concentration in the presence and in the absence of progesterone is plotted in Figure 4. The results clearly indicate that the observed inhibitions are due mainly to a direct effect on the catalytic activity of the enzyme. In order to examine in more detail the actions of estradiol and progesterone on the cytosolic cholesterol esterase, we investigate their effects on the kinetic behavior of the en-

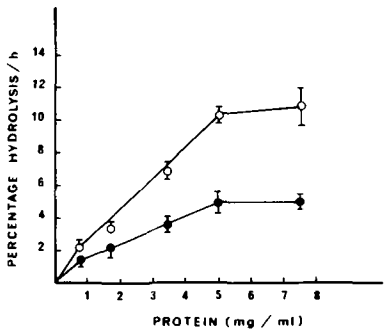

FIG. 4. Dependence of the rate of cholesteryl ester hydrolysis with protein concentration in the absence (o) and in the presence (•) of progesterone (50 μM). The hormone was added in 10 μl acetone. Control incubates contained 10 μl solvent. Each point is the mean of 4 determinations. Vertical bars represent the S.E. of the mean.

zyme. The apparent Michaelis & Menten values (Km) for cholesteryl oleate and the maximum velocities (V) were calculated by using a linear regression program and are listed in Table 2. The double reciprocal plots (L-B representation) of the data, shown in Figure 5, indicate that the addition of estradiol and progesterone raises the Km values for substrate; meanwhile, no concomitant changes in V are observed. The effects of estradiol and progesterone did not differ substantially from each other.

DISCUSSION

In the present paper we have examined the role of female sex hormones in the regulation of the hydrolysis of liver cytosol cholesteryl esters in order to assess whether the reported increases in this lipid in response to estradiol

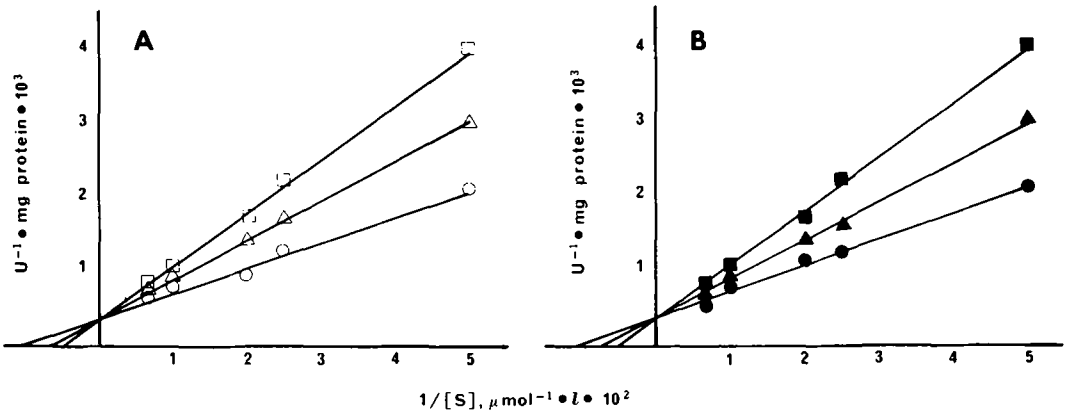

FIG. 5. Double-reciprocal plots of the inhibition kinetics of cytosolic cholesterol ester hydrolase from rat liver by estradiol and progesterone. A - assays contained estradiol as follows: o, none; △, 20 μM and □, 100 μM. B - assays contained progesterone as follows: •, none; ▲, 20 μM and ■, 100 μM. U, unit of enzyme; S, cholesteryl oleate. Each point is the mean of 3 determinations.

TABLE 2

Kinetic Data for the Inhibition of Cholesteryl Oleate Hydrolysis in Rat Liver Cytosol by Estradiol and Progesterone

Hormone added to the incubate	Km (μmol/l)	V (units/mg protein × 10^3)
None	99 ± 8	3.03 ± 0.22
Estradiol, 20 μM	155 ± 11*	3.11 ± 0.18
Estradiol, 100 μM	197 ± 16*	2.97 ± 0.25
Progesterone, 20 μM	139 ± 10*	3.18 ± 0.27
Progesterone, 100 μM	191 ± 18*	2.91 ± 0.13

Values were calculated from the Lineweaver & Burk plot by using a linear regression program with a microcomputer.
Data are the mean ± S.E. of 3 determinations.
*Significantly different from control.

and progesterone (6,16,17) would be due to a diminished cholesterol ester hydrolase activity. The results we have obtained with a single injection of estradiol are in part compatible with this presumption. The hydrolytic activity of the cytosolic fraction was significantly decreased after administration of the hormone, although this effect was detected only for a very short period (3-4 hr after injection). By contrast, progesterone was found to increase the liver enzyme activity, this effect also being achieved after a similar lag period of 4 hr. This observation and the fact that cholesteryl esters increased progressively between 4 and 24 hr following hormone injection clearly indicate that a causal relationship between esterase activity and hepatic lipid deposition cannot be assumed. Even the transient inhibition produced by the estradiol does not seem to account for the elevation in the level of cholesteryl esters. However, some interesting reflections can be deduced from these results.

The regulation of the enzyme cholesterol ester hydrolase has been studied intensively in those tissues where the free cholesterol level must be rigidly controlled. Several reports have demonstrated that cholesterol esterases are sensitive to hormonal stimuli in adrenal cortex (18-21), adipose tissue (22,23), brain (24), gonads (25) and aorta (26). Since liver is the major site for cholesterol and lipoprotein synthesis and the only site for the conversion of cholesterol to bile acids, it could be speculated that cholesterol ester hydrolase plays a more important role than simply hydrolyzing cholesterol esters from storage whereby it would be carefully modulated. The current level of knowledge about the regulation of cholesterol esterase in the liver of man or experimental animals and, moreover, the influence of gonadal steroids on that metabolic pathway, is poor. The present study shows that under our experimental conditions, estradiol and progesterone modulate cholesterol ester hydrolase activity resulting in early effects of opposite sign. This selectivity in esterase regulation is consistent with numerous other studies where progesterone has been shown to be quite an antagonist of estrogen action in several tissues (27-30). Both hormonal responses were abolished in 6-8 hr which might be due to the fact that hormones are lost from the tissue at a fast rate (31,32). An alternative explanation could be that liver cells adapt in some way to the continuing presence of the hormones, perhaps by altering either the sensitivity of the receptors or the hormone response pathway at some later stage.

Another aspect of the investigation reflects that estradiol and progesterone are capable of inhibiting the enzyme in a concentration dependent manner, since such decrease of the hydrolytic activity is not due to interactions between the hormone and the substrate. Both female sex hormones exerted similar actions on the kinetic properties of enzyme, so that marked increases in the apparent Km for cholesteryl oleate were found. The similarity among the structure of the hormones and substrate backs up the existence of a competition between them for the active site of the enzyme. The lack of response of esterase activity after 3 days of treatment, when the circulating hormone concentrations were elevated drastically, may be interpreted to mean that female sex hormones in vivo act through mechanisms more complex than simple competitive inhibitions. Furthermore, the discrepancies observed between the results from the in vivo and in vitro studies and the different responses induced by estradiol and progesterone suggest that they are, most probably, mediated by different mechanisms of action. It has been suggested that in rat uterus the estrogen-induced responses are mediated separately by independent mechanisms (33), among them the genomic reponses involving the cytosol-nuclear receptor system (34) and some other responses possibly involving the adenylcyclase (35) and/or guanylcyclase systems (36).

In conclusion, the results described in the present report provide substantial evidence that estradiol and progesterone are modulatory agents of the cytosolic cholesterol ester hydrolase in rat liver, although the biochemical basis underlying these regulatory effects is difficult to postulate.

REFERENCES

1. Deykin, D., and Goodman, D.S. (1962) J. Biol. Chem. 234, 68-74.
2. Stokke, K.T. (1972) Biochim. Biophys. Acta 280, 329-335.
3. Nilsson, A. (1976) Biochim. Biophys. Acta 450, 379-389.
4. Pittman, R.C., Attie, A.D., Carew, T.E., and Steinberg, D. (1979) Proc. Natl. Acad. Sci. USA 76, 5345-5349.
5. Stokke, K.T., and Norum, K.R. (1971) Scand. J. Clin. Lab. Invest. 27, 21-27.
6. Gandarias, J.M., Lacort, M., Ochoa, B., and Quiroga, M. (1981) Lipids 16, 449-453.
7. Gandarias, J.M., Lacort, M., and Ochoa, B. (1982) Steroids 39, 221-230.
8. Chao, Y., Windler, E.E., Chen, G.C., and Havel, R.J. (1979) J. Biol. Chem. 254, 11360-11366.
9. Owen, J.S. (1981) Nature 292, p. 106.
10. Kovanen, P.T., Brown, M.S., and Goldstein, J.L. (1979) J. Biol. Chem 254, 11367-11374.
11. Windler, E.E., Kovanen, P.T., Chao, Y., Brown, M.S., Havel, R.J., and Goldstein, J.L. (1980) J. Biol. Chem. 255, 10464-10471.
12. Faegerman, O., and Havel, R.J. (1975) J. Clin. Invest. 55, 1210-1218.

13. Andersen, J.M., Turley, S.D., and Dietschy, J.M. (1979) Proc. Natl. Acad. Sci. USA 76, 165-169.
14. Traynor, J.R., and Kunze, H. (1975) Biochim. Biophys. Acta 409, 68-74.
15. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
16. Hill, P., and Martin, W.J. (1972) Can. J. Biochem. 50, 474-483.
17. Gandarias, J.M., Abad, C., Lacort, M., and Ochoa, B. (1979) Rev. Esp. Fisiol. 35, 471-474.
18. Nishikawa, T., Mikami, K., Saito, Y., Tamura, Y., and Kumagai, A. (1981) Endocrinology 108, 932-936.
19. Yeaman, S.J., and Cook, K.G. (1980) FEBS Lett. 120, 212-216.
20. Cook, K.G., Lee, F.T., and Yeaman, S.J. (1981) FEBS Lett. 132, 10-14.
21. Pittman, R.C., and Steinberg, D. (1977) Biochim. Biophys. Acta 487, 431-444.
22. Khoo, J.C., Drevon, C.A., and Steinberg, D. (1979) J. Biol. Chem. 254, 1785-1787.
23. Pittman, R.C., Khoo, J.C., and Steinberg, D. (1975) J. Biol. Chem. 250, 4505-4511.
24. Arnaud, J., Nobili, O., and Boyer, J. (1981) Biochem. Biophys. Res. Comm. 100, 1167-1172.
25. Fitzpatrick, P., and Merum, K.M.J. (1979) Fed. Proc. 38, p. 230.
26. Subbiah, M.T.R. (1977) Steroids 30, 259-265.
27. Mukherjea, M., and Biswas, R. (1975) Endokrinologie 66, 113-121.
28. Weinstein, I., Turner, F.C., Soler-Argilaga, C., and Heimberg, M. (1978) Biochim. Biophys. Acta 530, 394-401.
29. Weinstein, I., Seltzer, M., and Belitsky, R. (1974) Biochim. Biophys. Acta 348, 14-22.
30. Bhakoo, H.S., and Katzenellenbogen, B.S. (1977) Mol. Cell. Endocrinol. 8, 105-120.
31. Pavlik, E.J., and Coulson, P.B. (1976) J. Steroid Biochem. 7, 369-376.
32. Katzenellenbogen, B.S. (1980) Ann. Rev. Physiol. 42, 17-35.
33. Tchernitchin, A. (1979) J. Steroid Biochem. 11, 417-424.
34. Jensen, E.V., and De Sombre, E.R. (1972) Ann. Rev. Biochem. 41, 203-230.
35. Singhal, R.L., and Lafreniere, R.T. (1972) J. Pharmacol. Explt. Therapeutics 180, 86-97.
36. Vesely, D.L., and Hill, D.E. (1980) Endocrinology 107, 2104-2109.

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Characterization of γ-Linolenic Acid in *Ribes* Seed

HELMUT TRAITLER,* HEIKE WINTER, URS RICHLI and YVES INGENBLEEK,
Nestlé Research Department, P.O. Box 88, CH-1814 La Tour de Peilz, Switzerland

ABSTRACT

The total lipid content of fruit seeds of the *Ribes* family ranges by weight from 18.3% in gooseberries *(Ribes uva crispa)* to 30.5% in black currants *(Ribes nigrum)*. Isolation procedures and analytical methods (gas chromatography, mass spectrometry, high performance thin layer chromatography and stereospecific analysis) demonstrate that the oils from *Ribes* seeds contain up to 19% by weight of γ-linolenic acid (γ-LA, C18:3, n-6) in black currant oil. This last *Ribes* species thus constitutes one of the richest natural sources in γ-LA yet described. These oils appear promising for critically ill patients who seem unable to convert linoleic acid into subsequent EFA fractions.
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INTRODUCTION

γ-linolenic acid (γ-LA, C18:3 n-6) is known to play a crucial step in the generation of prostaglandin derivatives (1). Under normal physiological circumstances, γ-LA results from the hepatic bioconversion of linoleic acid (LA, C18:2 n-6), the major essential fatty acid (EFA) for humans. The transformation of LA to the more unsaturated γ-LA requires the activation of the liver Δ6-desaturase enzyme (2). The dietary requirements for LA are estimated around 2.7% of the total caloric intake in children (3) and around 3-5 g/day in adults (4). These EFA amounts usually are supplied with a well-balanced diet. The endogenous conversion of LA into γ-LA and subsequent compounds proceeds normally, explaining why biochemical or clinical signs of EFA deficit are extremely rare.

In contrast, it is known that fat-free parenteral diet very rapidly exhausts the endogenous EFA resources, leading to biochemical and clinical abnormalities (5-8). Moreover, a number of recent reports suggest that the normal transformation of LA into further EFA fractions may be depressed under several stressful conditions (9-11), most probably as a result of the Δ6-desaturase depression (2). Critically ill patients thus become at risk of developing EFA-deficient status, even in the case of appropriate LA delivery. We have, therefore, focused our attention towards new lipid sources which could be of clinical usefulness in situations characterized by the enzyme defect. We were successful in isolating in the seeds of fruits belonging to the *Ribes* family varying γ-LA concentrations, amounting to 19% in black currant oil (12).

*To whom correspondence should be addressed at Nestlé Products Technical Assistance Co. Ltd., Research Department, P.O. Box 88, CH-1814 La Tour de Peilz, Switzerland.

ISOLATION PROCEDURES

In black currant varieties from Austria, France, Germany, Sweden and Switzerland, the amount of γ-LA varied within relatively narrow limits. Principally 3 different sources are available for the oil extraction from black currant seeds. These are: [1] whole fruits, including possibly enzymatic treatment for obtaining the seeds; [2] industrial residues (press cakes) from juice or jam production, and [3] industrial residues from fermentation processes, which are products of [2].

According to the degree of purification of the raw material, their fat content may vary between 13 and 30%, the latter being the amount of fat in washed grains. The press cake is milled and extracted on a Soxhlet apparatus with hexane to yield an oil which contains a large amount of wax and dyestuff, 7-8%. After their removal by winterization and bleaching, the resulting oil was analyzed.

Gas Chromatography (GC) Analysis

GC analyses of fatty acid methyl esters (FAMES) (13,14) were performed on tailor-made high-resolution Carbowax 20 M capillary columns (15,16). For this purpose the triglycerides were transmethylated with sodium methoxide (17,18). Figure 1 shows a typical GC of FAMES of black currant oil with the sequence: palmitic, stearic, oleic, elaidic, linoleic, γ-linolenic, α-linolenic, stearidonic and very little arachidic and gadoleic acid. The range of composition, according to the origin of the seeds, is shown in the legend. Each of these peaks was identified by retention time in comparison with known standards, except for stearidonic acid. Utilization of other stationary phases, like a mixture of OV-17 and SE-30 (1:1), and SP 2340, gave the same results.

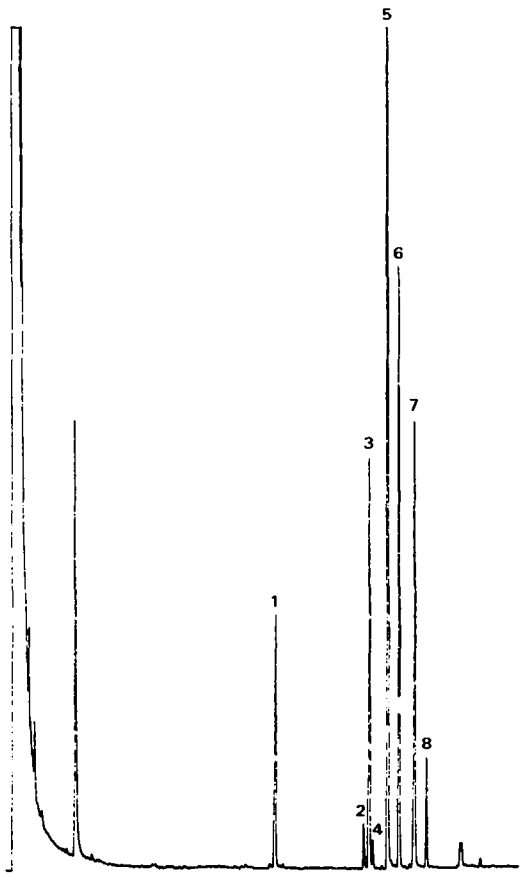
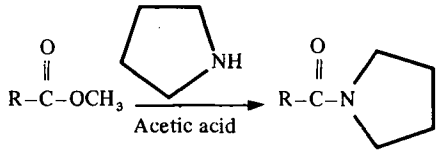


FIG. 1. GC of black currant oil FAMES. Conditions: Carlo Erba Mod. 4160 with high resolution Carbowax 20 M capillary column. On column injection, 80-230 C. For details, see references 15 and 16.

Peak number	FAMES	% of total lipid FAME
1	C16:0	6-7
2	C18:0	1-2
3	C18:1 N-9 *cis*	9-10
4	C18:1 N-9 *trans*	0.5
5	C18:2 N-6 all *cis*	47-49
6	C18:3 N-6 all *cis* (γ-linolenic acid)	15-19
7	C18:3 N-3 all *cis*	12-14
8	C18:4 N-3 all *cis* (stearidonic acid)	3-4
U	unknown (from solvent)	

Gas Chromatography/Mass Spectrometry (GC/MS) Analysis

Electron impact mass spectra of FAMES do not give structural information on double bond positions (19-24). To obtain these data FAMES or triglycerides may be converted into pyrrolidides (25).



The yield of this reaction under the described conditions is slightly over 90% and still some unreacted FAMES remain in the mixture. Our works demonstrated almost 100% conversion into the pyrrolidides when triglycerides were used as substrate instead of FAMES. Obviously, glycerol is a better leaving group than the methoxyl during substitution. A typical procedure was: 10 mg oil, 1 ml pyrrolidine and 0.1 ml acetic acid were stirred for 30 min at 100 C in a closed, round bottomed flask. Unreacted solvents were evaporated on a vacuum evaporator for about 30 min at 90 C bath temperature. The residue was taken up in heptane to give a 0.05% solution of which 1 μl was injected for GC/MS. However, GC-retention indices of the pyrrolidides are different from those of the methyl esters. The pyrrolidides of linoleic and γ-linolenic acid show very similar retention behavior on Carbowax columns and thus are very difficult to separate. This problem becomes even more pronounced in case of GC/MS, as peak broadening effects due to the interface result in reduced resolutions. On the other hand, α-linolenic pyrrolidide is well separated from the 2 n-6 acids 18:2 and 18:3, but shows the same resolution problem with stearidonic pyrrolidide 18:4 n-3. The basic separation principle of different compounds on Carbowax columns is related to their polarity. It appears that in the case of fatty acid pyrrolidides the polarity of the compounds is highly influenced by the positions of the double bonds, in particular those of $\Delta 6$ and $\Delta 9$, respectively, relative to the amide group, Figures 2a and 2b show the corresponding mass spectra of the pyrrolidides of γ-linolenic and stearidonic acids, respectively.

Fragmentation patterns of the different compounds show the exact positions of double bonds in each of the acids. The amide group obviously has a charge stabilization effect upon the fatty acid moiety (26-28) which results in more stable and characteristic fragments. According to the general rule, fragment intervals of 12 mass units which occur between the most intensive peaks of clusters of fragments containing n and n-1 carbon atoms of the acid moiety, indicate the double bond being located between carbons n and n+1. Accordingly, the EI-mass fragmentation of γ-linolenic pyrrolidide shows intervals of 12 mass units between carbons 5 and 6, 8 and 9 and 11 and 12, respectively

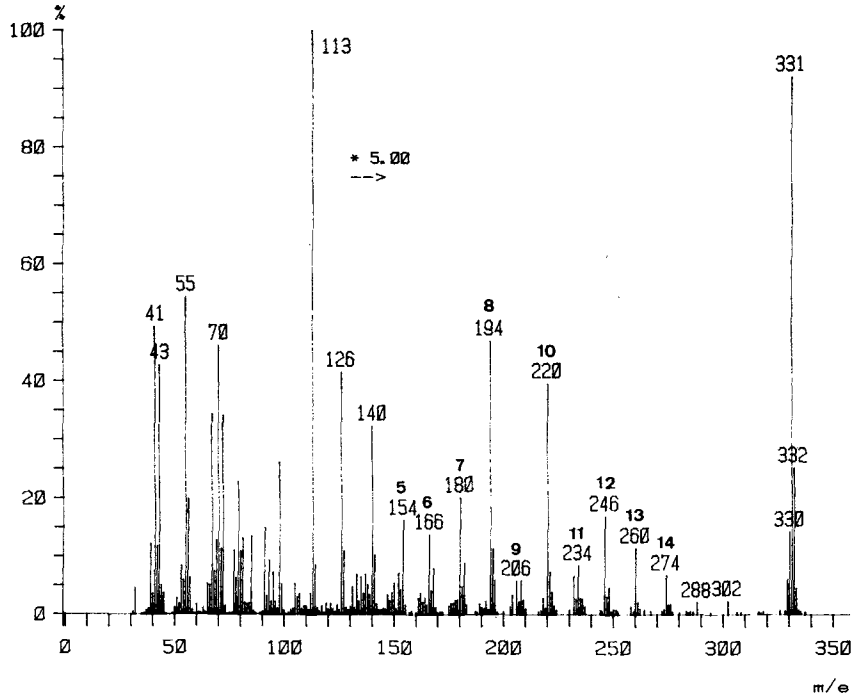


FIG. 2a. MS of N-octadec-6,9,12-trienoylpyrrolidide. Conditions: EI ionization 70 eV, 200 C (Kratos MS 30).

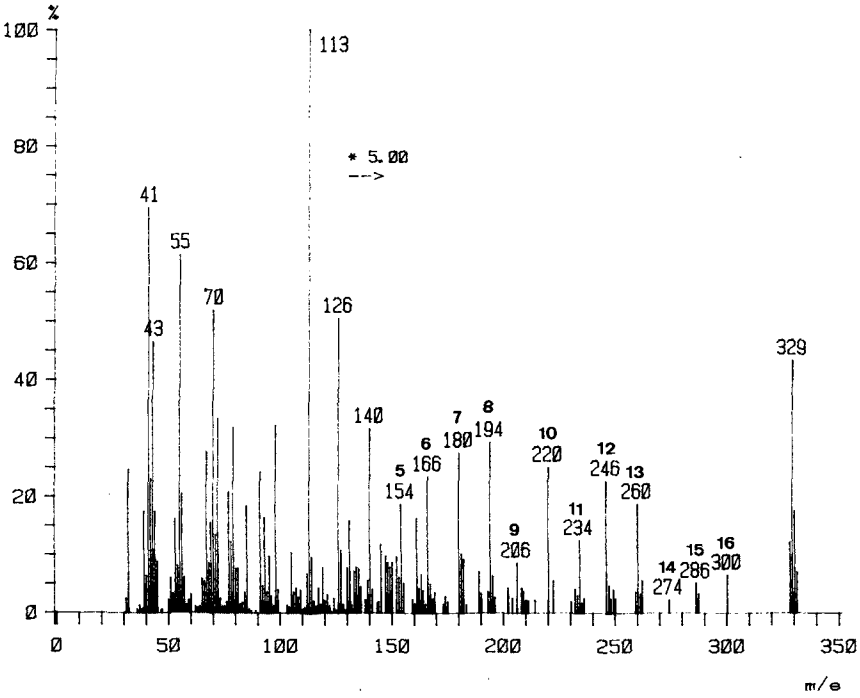


FIG. 2b. MS of N-Octadec-6,9,12,15-tetraenoylpyrrolidide. Same conditions as in Figure 2a.

(Fig. 2a). The presence of stearidonic acid C18:4, n-3 in black currant oil also could be proved by this method (Fig. 2b).

Preparative High Performance Thin Layer Chromatography (HPTLC)

As already mentioned above, GC separation of some of the fatty acid pyrrolidides was not sufficient for the production of well resolved peaks and clearly defined mass spectra. Therefore, a preparative preseparation of FAMES on thin layer plates had to be performed. Black currant oil was saponified and methylated with diazomethane in ether and a group separation according to the degree of unsaturation was achieved on reversed phase RP-18 as well as RP-12 HPTLC plates (A. Studer and H. Traitler, in preparation). Argentation chromatography which basically would lead to the same results was abandoned due to its inconvenience. Moreover, separation efficiencies are not better with the latter and quantification by visible scanning over the spots is practically impossible in the case of $AgNO_3$. Linoleic acid could be separated from γ- and α-linolenic acid, which migrated together, and stearidonic acid (18:4, n-3), the latter giving a single and very pure spot. Figure 3 shows a chromatogram scanned over a FAMES separation on RP-18 HPTLC plates. Conversion of the fractionated FAMES to the pyrrolidides gave ideal samples for further GC/MS analysis.

Analysis of Geometric Isomers

In order to obtain evidence on the stereoisomerism of the double bonds of the black currant oil fatty acids, an enzymatic analysis with lipoxygenase was performed (29-33). For this purpose 120 mg of oil were saponified with ethanolic potassium hydroxide solution; borate buffer and 0.5 N hydrochloric acid were added. After 20 min, an aliquot of this solution together with inactivated lipoxygenase was used for zero adjustment. Samples were measured with active lipoxygenase after a reaction time of 20 min. This method allows the determination of the total amount of *cis* double bonds in isolated pentadienoic structures and can be used for the quantification of *cis* double bonds in polyunsaturated fatty acids as well. For this purpose, black currant oil FAMES were submitted to an enzymatic analysis. Only those polyunsaturated fatty acids having *cis* double bonds in $\Delta 9$ and $\Delta 12$ positions are susceptible to lipoxygenase catalyzed oxydation to hydroperoxide (Fig. 4). This compound shows a UV absorption maximum of 234 nm and thus can ideally be used for the quantitative determination of most polyunsaturated fatty acids also in complex mixtures.

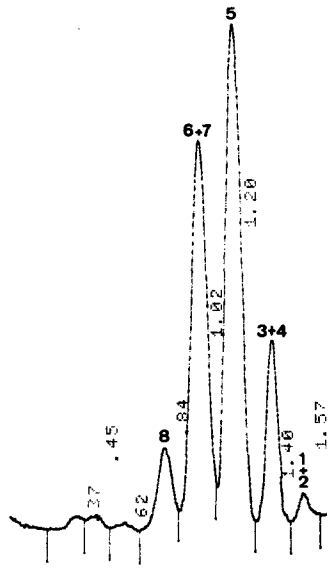


FIG. 3. HPTLC densitogram of black currant oil FAMES. Conditions: Nanoplate RP 18 (Merck) with solvents methanol/acetonitrile 1:1 dipping into 5% phosphomolybdic acid in acetone, charring at 150 C, 5 min. Scanning: Camag scanner, 546 nm, scanning speed 0.5 mm/sec. slit width 5 mm. Integration: Spectra Physics 4100, chart speed 4 cm/min, attenuation 128. (For peak numbers, see Fig. 1.) Vertical figures indicate retention times as printed out by the integrator.

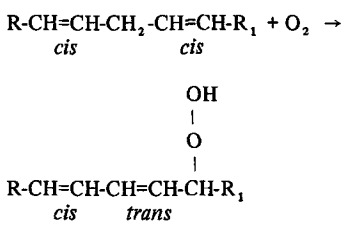


FIG. 4. Schematic pathway of lipoxygenase reaction.

The total amount of polyenic *cis-cis* compounds in black currant oil as determined gas chromatographically was 81.9%. Application of the enzymatic method, including the calibration with pure linoleic acid, gave a value of 83.7% polyunsaturated fatty acids. The conclusion can be drawn from these results that all polyunsaturated fatty acids in black currant oil also have all-*cis* double bonds.

DISCUSSION

The present study unequivocally demon-

TABLE 1

Linoleic Acid, γ-Linolenic Acid, Dihomo-γ-Linolenic Acid and Arachidonic Acid in Some Natural Products

Natural sources	Total lipid content	EFA fractions			
	g/100 ml (milks) g/100 g (seeds)	LA[b]	γ-LA[c]	DHγLA[d]	AA[e]
		(% of the total lipid content)			
Human colostrum[a]	2.5-3.5	7.82	0.34	0.49	0.71
Human mature milk[a]	3.5-4.5	10.75	0.35	0.31	0.41
Hops seeds (Humulus lupulus)	7.0	52.8	3-4	0	0
Hemp seeds (Cannabis sativa)	38.0	56.4	3-6	0	0
Red Currant Seeds (Ribes rubrum)	25.2	41.5	4-6	0	0
Evening Primrose Seeds (Oenothera biennis)	17	71.5	7-9	0	0
Gooseberry seeds (Ribes uva crispa)	18.3	40.0	10-12	0	0
Black Currant Seeds (Ribes nigrum)	30.5	48.5	15-19	0	0

[a]Values from Gibson and Kneebone, 1981 (ref. 36).

[b]LA = linoleic acid, C18:2 n-6.

[c]γ-LA = γ-linolenic acid, C18:3 n-6.

[d]DHγLA = dihomo-γ-linolenic acid, C20:3 n-6.

[e]AA = arachidonic acid, C20:4 n-6.

strates the presence of γ-LA in several fruit seeds belonging to the *Ribes* family, with the richest concentration reaching up to 19% of the total lipid content in seeds of black currant oil. This seed oil also contains 13.5% of α-linoleic acid (α-LA, C18:3 n-3) and 3.5% of the unusual stearidonic acid (SA, C18:4 Δ6,9,12,15 n-3) fraction, setting black currant oil in a very original position as regards EFA sources. Both α-LA and SA belong to the n-3 PUFA series leading to the formation of n-3 eicosapentaenoic acid (EPA, C20:5 n-3) and of docosahexaenoic acid (DHA, C22:6 n-3), a bioconversion requiring the same desaturases and elongases as those involved in the generation of n-6 and n-9 compounds. The n-3, n-6 and n-9 anabolic pathways are, therefore, in competition for the elaboration of their end-products. A recent patent (12) covers the fruit seeds of the *Ribes* family as purveyors of the physiologically active n-6 EFA fractions.

Healthy newborns submitted to breast-feeding are not at risk of developing signs of EFA deficit. Depending on maternal diet and nutritional status, large fluctuations in the LA content of human milk are described (34). Under normal circumstances, however, the LA concentration of human colostrum and milk lies within relatively narrow limits oscillating from 7% to 11% by weight of the total lipid content (35, 36). This means that LA intake by breast-fed children is significantly higher than that supplied by a well-balanced diet during any later period of life. The additional presence of γ-LA, dihomo-γ-linolenic acid (DHγLA, C20:3 n-6) and AA in human colostrum and milk (35,36) points to the uniqueness of breast-feeding and further suggests that EFA compounds fulfill special requirements in the growing child during the first months of life. Table 1 compares some EFA characteristics of human milk, 3 already described vegetable oils and 3 lipid fractions extracted from *Ribes* seeds.

Body EFA resources are very low at birth (1) so that fat-free enteral or parenteral diet causes biochemical and clinical abnormalities both in premature infants (5) and in healthy newborns (6,7). Biochemical signs of EFA-deprivation arise as early as one week after the onset of fat restriction (7,37) whereas clinical signs, such as dermatitis, usually develop after 3 weeks (38). Even adult patients with appropriate reserves of linoleate in adipose tissue triglycerides may exhibit early biochemical and clinical signs of EFA depletion (8,39). This situation typically is delineated by a gradual decrease in the long chain n-6 derivatives, contrasting with a progressive increase in endogenous FA substitutes for carbon chain elongation and desaturation. Theoretically, administration of lipid emulsions based on soybean oil or safflower oil would suffice to prevent biochemical and clinical symptoms of EFA deficiency, since these 2 vegetable products contain

54% and 77.5% LA, respectively. An increasing number of recent studies, however, demonstrate that use of emulsions containing LA may not solve the problem of EFA deficiency in total parenteral nutrition (TPN), since the blood and tissues of those patients contain significantly higher LA but lower arachidonate levels, a metabolic profile strongly suggestive of depressed bioconversion (6,7,8), entailing the accumulation of LA. Moreover, the major PG_1 and PG_2 urinary metabolites significantly decline during the course of TPN (9) to reach nadir values reminiscent of EFA deficient status (40). The most likely explanation resides in alterations of the $\Delta 6$-desaturase activity which seems reset at novel functional thresholds favoring the generation of n-9 derivatives at the expense of n-6 compounds. The current consensus is that the $\Delta 6$-desaturation is the rate limiting step in linoleic acid metabolism (41,42) and that the enzymatic activity may be inhibited by a number of factors such as protein restriction (43), fasting, premedication and anesthesia (44), and several nutritional or hormonal alterations (2) that are involved in the stress reaction (45). These data suggest that currently available lipid emulsions, despite their helpful supply of energy, may well not optimally fulfill the true EFA requirements of critically ill patients who, therefore, appear as major candidates for the beneficial effects of γ-LA supplementation.

REFERENCES

1. Aaes-Jorgensen, E. (1961) Physiol. Rev. 41, 1-51.
2. Brenner, R.R. (1977) in Function and Biosynthesis of Lipids. Vol. 83, pp. 85-101, Adv. Exp. Med. Biol. Plenum Press, New York.
3. American Academy of Pediatrics, Committee on Nutrition (1976) Pediatrics 57, 278-285.
4. Hassam, A.G., Rives, J.P., and Crawford, M.A. (1977) J. Nutr. 107, 519-524.
5. Panos, T.C., Stinnett, B., Cross, E., and Beard, A.G. (1961) Fed. Proc. 20, 366-372.
6. Hansen, A.E., Wiese, H.F., Boelsche, A.N., Haggard, M.E., Adam, D.J., and Davis, H. (1963) Pediatrics 31, 171-192.
7. Friedman, Z., Danon, A., Stahlman, M.T., and Oates, J.A. (1976) Pediatrics 58, 640-649.
8. McCarthy, M.C., Cottam, G.L., and Turner, W.W. Jr. (1981) Am. J. Surg. 142, 747-751.
9. Friedman, Z., and Fröhlich, J.C. (1979) Pediatr. Res. 13, 932-936.
10. Heird, W.C. (1983) Nutritional Support of the Pediatric Patient in Nutritional Support of the Seriously Ill Patient (Winters, R.W., and Greene, H.L., eds.) Vol. 1, pp. 157-179, Academic Press, New York.
11. Stein, T.P., Marino, P.L., Harner, R.N., Schluter, M.D., Leskiw, M.J., and Black, S. (1983) J. Am. Coll. Nutr. 2, 241-247.
12. Traitler, H., and Winter, H. Compositions nutritives contenant des corps gras et procédé de préparation de ceux-ci. European Patent application, published 26.10.1983, EUR 92085.
13. Marinetti, G.V. (1967) Lipid Chromatographic Analysis, Vol. 1, Marcel Dekker, New York.
14. Christie, W.W. (1973) Lipid Analysis, Pergamon Press, Oxford.
15. Traitler, H. (1983) J. High Resol. Chromatogr. & Chromatogr. Comm. 6, 60-63.
16. Traitler, H., Kolarovic, L., and Sorio, A. (1983) J. Chromatogr. 279, 69-73.
17. Shehata, A.Y., De Man, M.J., and Alexander, J.C. (1970) Can. Inst. Food Technol. J. 3, 85-89.
18. Badings, H.J., and De Jong, C. (1983) in Proceedings of the 5th Internat. Symp. Capillary Chromatogr. (Rijks, G., ed.), Elsevier, Amsterdam.
19. Sharkey, A.G. Jr., Schulz, J.L., and Friedel, R.A. (1959) Anal, Chem. 31, 87-94.
20. Ryhage, R., and Stenhagen, E. (1959) Arkiv Kemi 13, 523-542.
21. McLafferty, F.W. (1959) Anal. Chem. 31, 82-87.
22. Ryhage, R., Staellberg-Stenhagen, S., and Stenhagen, E. (1961) Arkiv Kemi 18, 179-194.
23. Hallgren, B., Ryhage, R., and Stenhagen, E. (1959) Acta Chem. Scand. 13, 845-847.
24. McFadden, W. (1973) Techniques of Combined Gas Chromatography, Mass Spectrometry, Wiley-Interscience, New York.
25. Vetter, W., Walther, W., and Vecchi, M. (1971) Helv. Chim. Acta 54, 1599-1605.
26. Andersson, B.A., and Holman, R.T. (1974) Lipids 9, 185-190.
27. Andersson, B.A., Christie, W.W., and Holman, R.T. (1975) Lipids 10, 215-219.
28. Takagi, T., and Itabashi, Y. (1982) Lipids 17, 716-723.
29. Holman, R.T., Egwim, P.O., and Christie, W.W. (1969) J. Biol. Chem. 244, 1149-1151.
30. MacGee, J. (1959) Anal. Chem. 31, 298-302.
31. Hamberg, M., and Samuelsson, B. (1967) J. Biol. Chem. 242, 5329-5335.
32. Dolev, A., Rohwedder, W.K., and Dutton, H.J. (1967) Lipids 2, 28-32.
33. Battaglia, R., and Mitiska, J. (1982) Mitt. Gebiete Lebensm. Hyg. 73, 35-43.
34. Cuthbertson, W.F. (1976) Am. J. Clin. Nutr. 29, 559-568.
35. Hall, B. (1979) Am. J. Clin. Nutr. 32, 304-312.
36. Gibson, R.A., and Kneebone, G.M. (1981) Am. J. Clin. Nutr. 34, 252-257.
37. Postuma, R., Peace, P.W., Watts, R., Taylor, S., and McEvoy, F.A. (1978) J. Pediatr. Surg. 13, 393-398.
38. Caldwell, M.D., Jonsson, H.T., and Othersen, H.B., Jr. (1972) J. Pediatr. 82, 894-898.
39. Wene, J.D., Connor, W.E., and DenBesten, L. (1975) J. Clin. Invest. 56, 127-134.
40. Friedman, Z., Seyberth, H., Lamberth, E.L., and Oates, J. (1978) Pediatr. Res. 12, 711-714.
41. Mead, J.F. (1971) in Progress in the Chemistry of Fats and Other Lipid (Holman, R.T., ed.), pp. 161-192, Pergamon Press, Oxford.
42. Crawford, M.A., Hassam, A.G., and Rivers, J.P. (1978) Am. J. Clin. Nutr. 31, 2181-2185.
43. De Tomas, M.E., Mercuri, O., and Rodrigo, A. (1980) J. Nutr. 100, 595-599.
44. Gómez Dumm, I.N., and Brenner, R.R. (1975) Lipids 10, 315-317.
45. Wilmore, D.W. (1983) J. Am. Coll. Nutr. 2, 3-13.

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Cholesterol Blocks the Disordering Effects of Ethanol in Biomembranes

JANE H. CHIN* and DORA B. GOLDSTEIN, *Department of Pharmacology, Stanford University School of Medicine, Stanford, CA 94305*

ABSTRACT

To assess the relation between the physical order of a membrane and its sensitivity to ethanol, we enriched biomembranes with cholesterol, both in vivo and in vitro. Japanese quail of the SEA line (selectively bred for susceptibility to experimental atherosclerosis) were treated for 9 to 16 weeks with a diet that contained 2% cholesterol. This regimen increased the cholesterol content of serum and erythrocytes. The cholesterol content of brain synaptosomal plasma membranes (SPM) was unaffected by the high cholesterol diet. In other experiments, isolated mouse synaptosomal plasma membranes were incubated with cholesterol/phospholipid (C/P) vesicles; different amounts of cholesterol were transferred according to the sterol content of the donor vesicles. Membrane order was determined in both types of membranes by a sensitive electron paramagnetic resonance (EPR) technique. The order parameter with 5- and 12-doxylstearic acid increased along with the cholesterol content. As expected, ethanol disordered membranes (decreased the order parameter) in a concentration-related manner. The slope of the concentration response curve was less steep in high cholesterol than low cholesterol membranes, indicating that cholesterol enrichment partially blocks the membrane action of ethanol in both types of membranes.

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INTRODUCTION

The ratio of cholesterol and phospholipids in cell membranes is an important determinant of the fluidity of the bilayer. The cholesterol content of biomembranes can be altered experimentally in some tissues by dietary means (1) or in vitro by incubation with cholesterol/phospholipid (C/P) dispersions (2). Upon insertion into the membrane, cholesterol orients its rigid sterol ring with the 3β-hydroxyl group toward the aqueous phase and its flexible hydrocarbon chain toward the center of the bilayer (3). At physiological temperatures the addition of cholesterol orders fluid lipid bilayers (4).

In contrast to cholesterol, alcohols disorder the structure of membrane bilayers (5-10). Sensitive electron paramagnetic resonance (EPR) or fluorescence polarization techniques show that sublethal concentrations of ethanol disorder many types of biomembrane, including synaptosomal plasma membranes (SPM) (5,6), erythrocyte membranes (5), and hepatic mitochondrial membranes (8). The effect is seen in the absence of proteins, e.g. in lipid extracts of biomembranes (6-8). This observation suggests that ethanol affects the lipids themselves and that the chemical composition of the membrane lipids might control their response to ethanol. That the differential sensitivity to ethanol in individual types of biomembranes (5,6) might be due to their different cholesterol content is suggested by the relative insensitivity of cholesterol-rich myelin membranes compared to SPM (5,6) and by our previous demonstration (9) that the disordering of ethanol in phosphatidylcholine liposomes decreases progressively with increasing cholesterol content. Cholesterol cannot be the only factor, however, because lipids extracted from SPM can differ in sensitivity to ethanol even when their cholesterol content has been made equal (7). Differences in other membrane components (10) as well as differential interaction of these compounds with cholesterol in microdomains (11) may account for these findings.

We report here the results of enriching biomembranes with cholesterol in vivo and in vitro. The intrinsic order increased with the C/P molar ratio, and high cholesterol membranes were relatively resistant to the disordering effect of ethanol.

METHODS

Quail Erythrocyte and Brain Membranes

Erythrocyte membranes and brains from male Japanese quail of the SEA line, selectively bred to be susceptible to experimental atherosclerosis (12), were provided by Dr. Keith Lurie. As reported elsewhere (13), one group of quail was fed for 6-16 weeks on a diet containing 2% cholesterol in 50% wild bird seed and

*To whom correspondence should be addressed.

50% mash. A control group received the same diet without added cholesterol. The quail were killed in pairs (one experimental and one control bird) after different lengths of time on the diet.

Quail whole brains were used to prepare SPM (14) for cholesterol and phospholipid analyses. Erythrocyte membranes were prepared by the method of Hoffman et al. (15), slightly modified. The red cell membrane suspension in 50 mM TRIS buffer pH 7.5 with 10 mM $MgCl_2$ was centrifuged at 500 × g for 2 min to remove any remaining debris, and the supernatant was used within 24 hr for EPR experiments.

Mouse Brain Membranes

SPM were prepared by the method of Jones and Matus (14) from whole brains pooled from 12 to 27 male Swiss Webster mice (8-10 weeks old, Charles River Breeding Laboratories, Wilmington, Massachusetts). The membrane fraction collected at the interface between 28.5 and 34% (w/w) sucrose layers was diluted with cold phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4) and recovered by centrifugation at 27000 × g for 20 min. The membranes were resuspended in PBS and stored at −80 C under nitrogen or used immediately for lipid exchange.

The SPM were enriched with cholesterol by incubating them with cholesterol/phosphatidylcholine vesicles. The purity of cholesterol (Sigma Chemical Co., St. Louis, Missouri) and egg phosphatidylcholine (Calbiochem, La Jolla, California) was checked by thin layer chromatography (TLC) in chloroform/methanol/water (65:25:4, v/v/v) and in benzene/ethyl acetate (3:2, v/v), respectively. Each compound was detected as a single spot upon exposure to iodine or after charring with sulfuric acid. The lipids were dissolved in chloroform/methanol (3:2, v/v) at ratios of 0.6 to 1.0 mole cholesterol per mole phospholipid and spread on the sides of a flask by evaporating the solvent with a stream of nitrogen; any remaining solvent was removed under vacuum overnight. The lipid films were hydrated with PBS to a concentration of about 2.7 mg of phosphatidylcholine/ml, and the membrane suspension was sonicated under a nitrogen atmosphere until clear. Usually a Heat Systems Sonicator was used with a ½-inch tip probe and a circulating cell immersed in ice water to prevent overheating, but in a few experiments a bath sonicator was used. To minimize adsorption of intact vesicles to the membranes (16), the lipid suspension was diluted after sonication with a solution of bovine serum albumin to a final concentration of 0.75% protein and 0.2% phospholipid. The dispersions were centrifuged at 106,500 × g for 60 min to remove undispersed lipid and titanium fragments from the sonicator probe tip. The supernatant was used for incubation with membranes or for lipid analyses.

SPM were incubated with the vesicles in 50-ml Erlenmeyer flasks at 37 C with shaking. Aliquots of membrane suspension in PBS (one ml containing 4 mg protein) were mixed with two ml of liposome-albumin sonicate or PBS. The final incubation medium contained 100 U/ml of penicillin and 100 μg/ml of streptomycin (Pen-Strep, Irvine Scientific Co., Santa Ana, California). After 12-20 hr of incubation under a nitrogen atmosphere, the membranes were separated from the liposomes by layering onto 0.5 M sucrose and centrifuging at 100,000 × g for 40 min (17). The resultant SPM pellet was washed 4 times with glass-distilled water (for lipid analyses) or with PBS (for EPR experiments). The washed membranes were resuspended in glass-distilled water or PBS, flushed with nitrogen, and stored at −80 C.

EPR Measurements

Mouse SPM that had been enriched with cholesterol in vitro and stored frozen were thawed, diluted with PBS to a protein concentration of 20 mg/ml, and dispersed by sonication in a Heat Systems bath disruptor horn. Freshly prepared erythrocyte membranes were concentrated by centrifugation, and most of the supernatant was removed. Both types of membranes were spin-labeled with 5- or 12-doxylstearic acid at 37 C as previously described (9). SPM were incubated for 30 min at a probe concentration of about 0.3 mM (6 to 8 μg/mg of protein). The mean spin label concentration in the erythrocyte membrane suspension was about 0.3 to 0.4 mM. Buffer or diluted ethanol was added to the spin-labeled membranes to a final concentration of 0, 87.5, 350 or 700 mM ethanol. The final protein concentration in brain membranes was 17 mg/ml.

Order parameters (18) were determined by a smoothed computer curve fit of the relevant peaks of the recorded spectra, as previously described (9). The order parameter for a single sample was the mean of the values determined from each of 3 8-min scans. Two to 4 samples were run for each concentration of ethanol.

Lipid Analyses

Lipids from SPM were extracted by the procedure of Folch et al. (19), modified as previously described (20) except that the final potassium citrate wash was omitted. Total lipids were extracted from liposomes with 20 volumes of chloroform/methanol (2:1) and were washed with 0.2 volume of 0.05 M KCl. Lipids were assayed by spectrophotometric methods for cholesterol (21) and for total phospholipid phosphorus (22).

Statistical Analyses

Values for control and cholesterol-fed quail were compared by Student's t test for paired observations. The concentration-related disordering effect of ethanol as well as the ordering effect of cholesterol was determined from the slope of regression lines obtained from least-squares analyses. The difference between the slopes of 2 regression lines was assessed by t test if the F ratio of the variances was not significant.

RESULTS

Quail Membranes Enriched in vivo

As reported elsewhere (13), the sterol diet increased the cholesterol content of erythrocytes and serum from SEA Japanese quail. The elevation was maximal by 8 weeks of treatment; no further change occurred up to 16 weeks. The serum cholesterol levels increased 6 to 7 times and the C/P molar ratio in erythrocyte membranes rose about 28% (Table 1), due mainly to an increase in cholesterol content. In contrast, the C/P molar ratio in brain membranes was essentially unaltered.

Cholesterol feeding significantly increased order parameters of quail erythrocyte membranes spin-labeled near the surface (5-doxylstearic acid probe) and in the interior of the bilayer (12-doxyl label) (Table 1).

Ethanol was added in vitro to red cell membranes from a group of quail that had been on the diet for 12-16 weeks. The C/P molar ratio of membranes from the cholesterol-fed birds in this group was 34% higher than that of their controls. The addition of ethanol decreased the order parameter in a concentration-related manner (Fig. 1). Both spin labels showed that membranes from the cholesterol-fed birds were more rigid than those of controls, both in the absence and presence of ethanol (Fig. 1). In each individual experiment the membranes from cholesterol-treated quail were less affected by ethanol than were membranes of the corresponding control bird tested on the same day. The slopes of the regression lines for individual birds in the cholesterol-fed group were less steep than those for the controls ($P < 0.05$ by t test for paired observations, Table 2).

Mouse Membranes Enriched in vitro

Isolated mouse SPM were enriched with cholesterol in vitro by incubation with phospholipid vesicles containing various proportions of cholesterol. Different amounts of cholesterol were transferred to SPM according to the sterol content of the donor vesicles (data not shown).

As expected, the order parameter was lower with the 12-doxyl probe than with the 5-doxyl probe when spectra were recorded at the same

TABLE 1

Effects of Diet on Cholesterol Content of Serum and of Erythrocyte and Brain Membranes (SPM) of Japanese Quail

	Controls	Cholesterol-fed
Serum		
Total cholesterol, mg/100 ml	142 ± 11	960 ± 91***
Erythrocyte membranes		
C/P molar ratio	0.584 ± 0.020	0.748 ± 0.018***
Order parameter, 5-doxyl, 37 C	0.617 ± 0.001	0.623 ± 0.002**
Order parameter, 12-doxyl, 19 C	0.578 ± 0.007	0.605 ± 0.010***
SPM		
C/P molar ratio	0.598 ± 0.011	0.605 ± 0.009

Each value is the mean ± S.E.M. of 7 birds (except N = 6 for serum cholesterol). The cholesterol-fed quail were maintained on a 2% cholesterol diet for 9 to 16 weeks. The controls received the same diet without the added cholesterol. Membranes were prepared from a bird in each group at about weekly intervals. These birds were part of a larger group whose serum and red cell (but not SPM) data are reported elsewhere (Lurie, K.G., Chin, J.H., and Hoffman, B.B., submitted for publication).

**$P < 0.01$ and ***$P < 0.001$ by Student's t test for paired comparisons with controls.

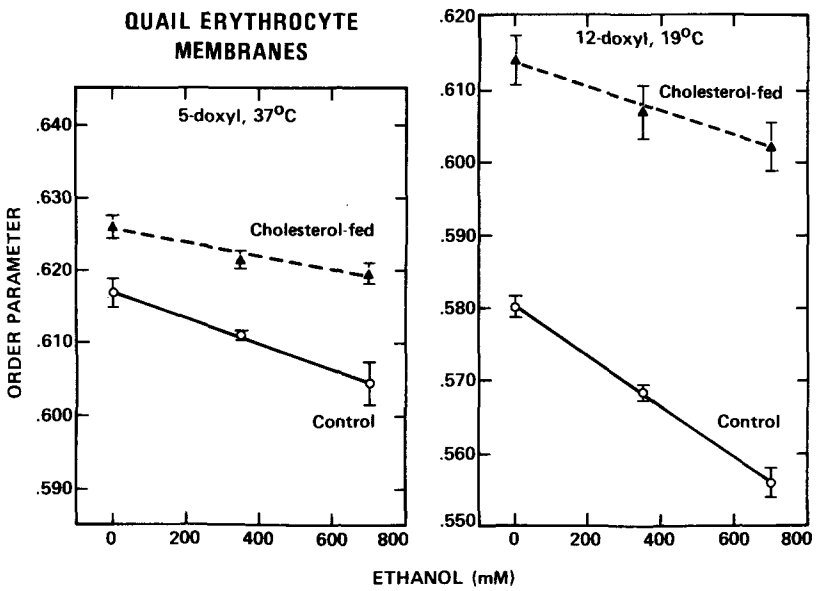

FIG. 1. Disordering effect of ethanol in erythrocyte membranes from cholesterol-fed quail. Ethanol was added in vitro to erythrocyte membranes from quail fed the cholesterol diet for 12 to 16 weeks and the corresponding control birds. Membranes from individual birds were spin-labeled and spectra were recorded at 37 C with 5-doxylstearic acid or at 19 C with 12-doxylstearic acid. Points represent means and vertical bars show S.E.M. for 3 birds.

TABLE 2

Effect of Cholesterol on the Response to Ethanol in vitro

C/P molar ratio	Decrease in order parameter per 500 mM ethanol	
	5-doxyl	12-doxyl
Quail erythrocyte membrane		
0.56	.009	.017
0.75	.005[a]	.009[a]
Mouse SPM		
0.66	.009	.021
0.75	.008	.016[b]
0.84	.006[c]	.012[c]

The C/P for the quail erythrocytes is the group mean for the control (0.56) and the cholesterol-fed (0.75) birds. The C/P for mouse SPM is that of different suspensions.

EPR data are the slopes of the lines in Figures 1 and 3, expressed as the decrease in order parameter per 500 mM ethanol. For quail, EPR spectra were recorded at 37 C with 5-doxyl and at 19 C with the 12-doxyl probe. For mouse, spectra were recorded at 28 C with both probes.

[a] $P < 0.05$ by Student's t test for paired comparisons of slopes between individual birds in the control and cholesterol-fed groups.

[b] $P < 0.05$ by t test vs. the slope when C/P = 0.66.

[c] $P < 0.05$ vs. the slope when C/P = 0.75 and $P < 0.001$ vs. the slope when C/P = 0.66.

temperature. Cholesterol increased the order parameter at both depths of the bilayer. As in quail erythrocyte membranes (13), the order parameter increased with the C/P molar ratio ($r = 0.87$, $P < 0.001$ for 5-doxyl and $r = 0.83$, $P < 0.001$ for the 12-doxyl probe) (Fig. 2). With 12-doxylstearic acid the effect of cholesterol was quite marked, as shown by the steep slope of the regression line. Expressed as the increase in order parameter per 0.1 unit increase in the C/P molar ratio, the slopes and 95% confidence intervals at 28 C were 0.011 (0.006 to 0.016) for 12-doxyl and 0.003 (0.002 to 0.004) for 5-doxylstearic acid.

The addition of ethanol to mouse SPM produced a concentration-dependent decrease in the order parameter that was quite pronounced with the 12-doxyl probe (Fig. 3). Cholesterol partially blocked the ethanol effect, as seen most easily with the 12-doxyl probe (Table 2). As Figure 4 shows, the higher the cholesterol, the smaller was the disorder produced by ethanol ($r = 0.96$, $P < 0.001$ for 12-doxyl; $r = 0.74$, $P < 0.06$ for 5-doxyl).

DISCUSSION

The cholesterol-enriched diet greatly increased the serum cholesterol in quail, and the sterol apparently was transferred from serum to

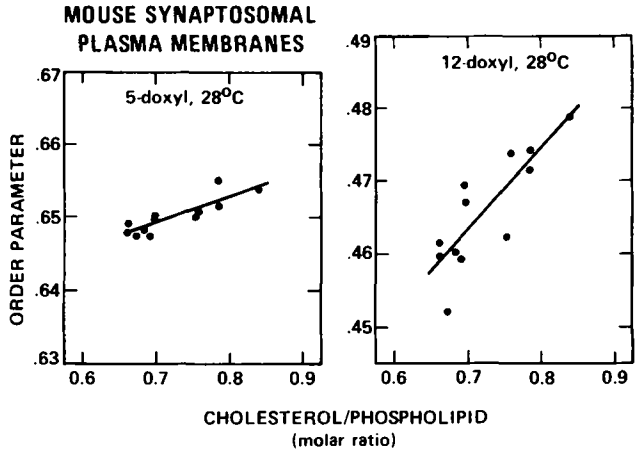

FIG. 2. Ordering effect of cholesterol in mouse SPM. Cholesterol was transferred in vitro to isolated mouse SPM by incubation with vesicles of different C/P molar ratio. A sample was taken for lipid assays and the membranes were then spin-labeled with 5- or 12-doxylstearic acid. Spectra were recorded at 28 C. Each point represents the mean order parameter determined from 2 to 4 samples.

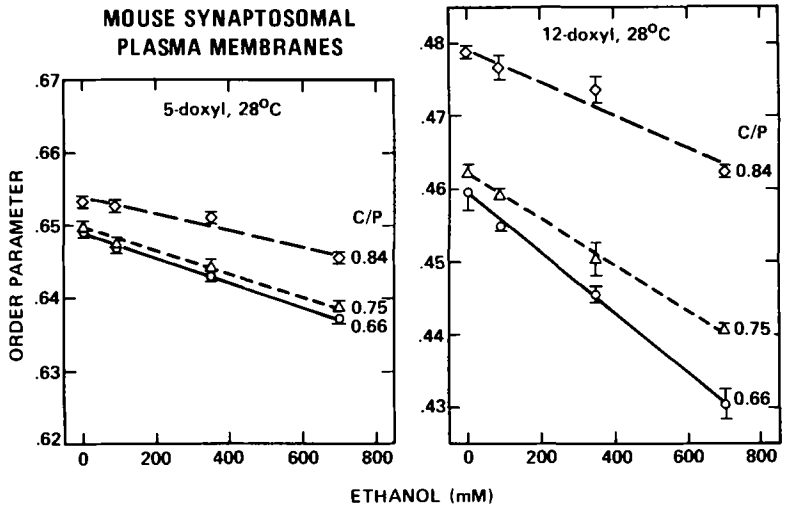

FIG. 3. Disordering effect of ethanol in mouse SPM enriched with cholesterol in vitro. Portions of a membrane preparation pooled from whole brains of 21 mice were enriched with cholesterol to different extents by incubation with liposomes, producing 3 suspensions of different cholesterol content. The lower line (C/P = 0.66) represents unincubated membranes. Ethanol was added to each of the 3 suspensions after adding 5- or 12-doxylstearic acid, and EPR spectra were recorded at 28 C. Each point represents the mean and range of the order parameters for 2 to 3 samples of each suspension at each ethanol concentration. The lines are calculated from least squares analyses. Numerical values of slopes are shown in Table 2.

erythrocyte membranes, a well-known process. Even though the membrane sterol content was increased, we could not predict with certainty that the membranes would become more rigid. This is because dietary changes are slow and there is ample opportunity for adaptive responses. The ordering effect of an unavoidable uptake of cholesterol might have been offset by

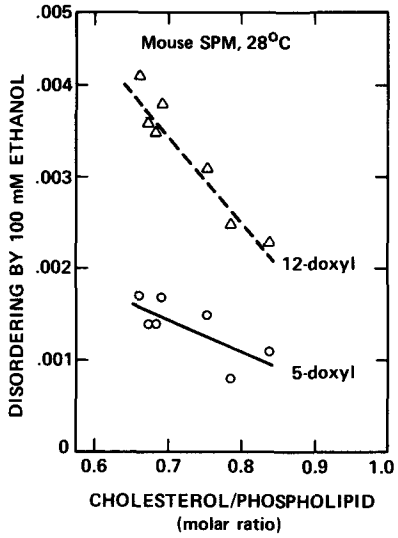

FIG. 4. Relation between membrane disordering by ethanol and membrane cholesterol content. The magnitude of membrane disordering is expressed as the absolute change in order parameter per 100 mM ethanol, determined from slopes of regression lines such as those shown in Figure 3.

an increased degree of unsaturation in the acyl chains of phospholipids, as has been shown in CHO cells (23). Apparently no such compensation occurred; we found the erythrocyte membranes of cholesterol-treated birds to be more ordered than those of controls. As previously shown (13), the order parameters measured with both 5- and 12-doxylstearic acid increased progressively with sterol content. In the current work, the same increase in order parameter with cholesterol content was seen in mouse SPM after incubation with cholesterol in vitro. These findings confirm the ordering effect of cholesterol shown by fluorescence polarization in human erythrocyte membranes (2) and in rat SPM that were depleted or enriched with cholesterol (17). Not all reports agree, however. Some workers (24,25) found that cholesterol decreased the microviscosity of SPM at temperatures below 37 C.

Brain, unlike other tissues, synthesizes its own cholesterol and does not exchange with plasma (26). However, since most of the brain cholesterol is in the myelin fraction, changes in the relatively small pool of sterol in neuronal plasma membranes might have been missed. In our experiments, SPM isolated from cholesterol-fed quail did not change their cholesterol content at all, even though they were perfused by serum that contained 7 times more cholesterol than normal. Nevertheless, SPM can take up cholesterol in vitro by a passive process, as shown here. Some protective mechanism must prevent this uptake in vivo.

Our data show that cholesterol greatly reduces motion in the middle region of phospholipid acyl chains, confirming results with EPR (9,27), fluorescence (28) and deuterium nuclear magnetic resonance (29) in phosphatidylcholine model membranes. It is not appropriate to compare the effects of cholesterol with 5- and 12-doxylstearic acid directly. The order parameter may not change linearly on addition of a perturbing agent. Thus, the magnitude of the change may depend on the initial value of the order parameter. Further, Taylor and Smith (30) postulated that the membrane may perturb the geometry of the doxyl group, especially when the oxazolidine ring is in the ordered region of the bilayer, near the surface. The distortion of the probe is thought to produce an artifactually low order parameter. Changes produced by cholesterol at the 5- (but not at the 12-) position might be partly offset by increased distortion of the probe, accounting for the smaller effect of cholesterol we observed with the 5-doxyl probe than with 12-doxylstearic acid.

Our previous studies showed that the disordering effect of ethanol in egg phosphatidylcholine vesicles was greatest in the absence of cholesterol and was blocked gradually as the cholesterol content was elevated (9). Although it was not obvious that complex biomembranes would respond in the same manner, we did observe in both quail erythrocyte and mouse brain membranes that the disordering effect of ethanol was greater at low than at high cholesterol content, especially with the 12-doxyl label. Thus cholesterol dampened the effects of ethanol in biomembranes in these EPR experiments, as also has been observed in rat SPM in fluorescence experiments with 1,6-diphenylhexatriene (DPH) (25). In contrast, other fluorescence polarization studies with DPH in lipid extracts, rather than intact membranes, suggest that some cholesterol may be needed for a maximal disordering effect of ethanol. Ethanol was more potent in the total lipid extract (without ganglioside) from SPM than in the phospholipid fraction alone (6). Similarly, Johnson et al. (7) found that the readdition of cholesterol to membrane lipid extracts from which it previously had been removed enhanced the disordering effect of ethanol. Cholesterol may interact with specific phospholipids (11) to induce lateral phase separations, and some of the microdomains thus formed may be especially sensitive to ethanol.

EPR and fluorescence studies agree that certain membranes isolated from chronically ethanol-treated animals are relatively resistant to the membrane-disordering effect of ethanol added in vitro (8,31-34). The chemical basis for this apparent compensatory response has not yet been determined. Cholesterol seems an appropriate additive for this purpose, and indeed it is sometimes (but not always) elevated by chronic ethanol treatment (35). Cholesterol may act by decreasing the partition coefficient of ethanol in membranes (32). However, other lipids also may change with chronic ethanol treatment (35).

In model membranes, ethanol reduces EPR order parameters most sharply at high temperatures and at low cholesterol levels, suggesting that disordered domains of biomembranes are particularly vulnerable. Thus, we might expect the alcohol to perturb membranes in regions where cholesterol is scarce, such as intracellular membranes and the immediate environment of some membrane-bound proteins. There is evidence that cholesterol is distributed unevenly in biomembranes (36) and that the C/P ratio may be lower in the lipid annulus of a membrane-bound protein than in the bulk lipid (37). If there exist some membrane proteins whose boundary lipids are cholesterol-poor, the functions of these proteins may be particularly susceptible to disruption by ethanol.

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REFERENCES

1. Cooper, R.A., Leslie, M.H., Knight, D., and Detweiler, D.K. (1980) J. Lipid Res. 21, 1082-1089.
2. Cooper, R.A., Leslie, M.H., Fischkoff, S., Shinitzky, M., and Shattil, S.J. (1978) Biochemistry 17, 327-331.
3. Huang, C-H. (1977) Lipids 12, 348-356.
4. Demel, R.A., and de Kruyff, B. (1976) Biochim. Biophys. Acta 457, 109-132.
5. Chin, J.H., and Goldstein, D.B. (1977) Mol. Pharmacol. 13, 435-441.
6. Harris, R.A., and Schroeder, F. (1981) Mol. Pharmacol. 20, 128-137.
7. Johnson, D.A., Lee, N.M., Cooke, R., and Loh, H.H. (1979) Mol. Pharmacol. 15, 739-746.
8. Waring, A.J., Rottenberg, H., Ohnishi, T., and Rubin, E. (1981) Proc. Natl. Acad. Sci. USA 78, 2582-2586.
9. Chin, J.H., and Goldstein, D.B. (1981) Mol. Pharmacol. 19, 425-431.
10. Harris, R.A., Groh, G.I., Baxter, D.M., and Hitzemann, R.J. (1984) Mol. Pharmacol. 25, 410-417.
11. Van Dijck, P.W.M. (1979) Biochim. Biophys. Acta 555, 89-101.
12. Day, C.E., Stafford, W.W., and Schurr, P.E. (1977) Lab. Animal Sci. 27, 817-821.
13. Chin, J.H., Lurie, K.G., Hoffman, B.B., and Goldstein, D.B. (1983) Fed. Proc. 42, 1772.
14. Jones, D.H., and Matus, A.I. (1974) Biochim. Biophys. Acta 356, 276-287.
15. Hoffman, B.B., Mullikin-Kilpatrick, D., and Lefkowitz, R.J. (1979) J. Cyclic Nucleotide Res. 5, 355-366.
16. Bloj, B., and Zilversmit, D.B. (1977) Biochemistry 16, 3943-3948.
17. North, P., and Fleischer, S. (1983) J. Biol. Chem. 258, 1242-1253.
18. Hubbell, W.L., and McConnell, H.M. (1971) J. Am. Chem. Soc. 93, 314-326.
19. Folch, J., Lees, M., and Stanley, G.H.S. (1957) J. Biol. Chem. 226, 497-509.
20. Chin, J.H., Parsons, L.M., and Goldstein, D.B. (1978) Biochim. Biophys. Acta 513, 358-363.
21. Rudel, L.L., and Morris, M.D. (1973) J. Lipid Res. 14, 364-366.
22. Morrison, W.R. (1964) Anal. Biochem. 7, 218-224.
23. Sinensky, M. (1980) J. Cell Biol. 85, 166-169.
24. Shapiro, H.K., and Barchi, R.L. (1981) J. Neurochem. 36, 1813-1818.
25. Crews, F.T., Majchrowicz, E., and Meeks, R. (1983) Psychopharmacology 81, 208-213.
26. Kabara, J.J. (1973) Prog. Brain Res. 40, 363-382.
27. Schreier-Muccillo, S., Marsh, D., Dugas, H., Schneider, H., and Smith, I.C.P. (1973) Chem. Phys. Lipids 10, 11-27.
28. Kutchai, H., Chandler, L.H., and Zavoico, G.B. (1983) Biochim. Biophys. Acta 736, 137-149.
29. Stockton, G.W., and Smith, I.C.P. (1976) Chem. Phys. Lipids 17, 251-263.
30. Taylor, M.G., and Smith, I.C.P. (1983) Biochim. Biophys. Acta 733, 256-263.
31. Chin, J.H., and Goldstein, D.B. (1977) Science 196, 684-685.
32. Rottenberg, H., Waring, A., and Rubin, E. (1981) Science 213, 583-585.
33. Lyon, R.C., and Goldstein, D.B. (1983) Mol. Pharmacol. 23, 86-91.
34. Harris, R.A., Baxter, D.M., Mitchell, M.A., and Hitzemann, R.J. (1984) Mol. Pharmacol. 25, 401-409.
35. Chin, J.H., and Goldstein, D.B., (In press) in Membrane Fluidity in Biology (Aloia, R.C. and Boggs, J.M., eds.) Vol. 3, Academic Press, New York.
36. Boggs, J.M. (1980) Can. J. Biochem. 58, 755-770.
37. Silvius, J.R., McMillen, D.A., Saley, N.D., Jost, P.C., and Griffith, O.H. (1984) Biochemistry 23, 538-547.

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Molecular Species and Fatty Acid Distributions of Triacylglycerols from Germinating Soybean Cotyledons

HIROMI YOSHIDA, *Department of Nutrition, Kobe-Gakuin University, Arise, Ikawadani-cho, Nishi-ku, Kobe 673, Japan*

ABSTRACT

Molecular species and fatty acid distributions of triacylglycerols obtained from cotyledons of soybean seedlings were investigated. Changes observed in triacylglycerol content were closely related to levels of total lipids present in the cotyledons. At day 12 of seedling growth, ca. 85% of triacylglycerols had been consumed. Immediately after the beginning of imbibition the oil consisted of triacylglycerols with even carbon numbers (from C-50 to C-60) based on the combined length of the fatty acyl chains present in a triacylglycerol. The dominant components throughout germination were C-52 and C-54 triacylglycerols. Fourteen molecular species of triacylglycerols were identified in the cotyledons. As soybean seedlings grew, the percentages of triacylglycerols decreased to 0.9-36.2% during the 12 days. Triacylglycerols containing one or more saturated fatty acids were hydrolyzed slightly faster than other species. Unsaturated fatty acids were dominant in the 2-position throughout germination. These results suggest the mechanism of initial triacylglycerol hydrolysis may be different in various molecular species.

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INTRODUCTION

Natural triacylglycerols are known to be made up of a complex mixture of molecular species (1-3), which are believed to influence the biological properties for mobilization in storage tissues during germination (4,5). Alteration of triacylglycerol composition of oil seeds during maturation is being investigated for several crops (6-8), and it is now possible to define with precise conversion reactions a number of precursors to triacylglycerols (9,10). In these crops, there is genetic variability in how fatty acid concentration affects the triacylglycerol species formed (11). During seedling growth of oil seeds, the reserve triacylglycerols in the storage tissues are first hydrolyzed to fatty acid and acylglycerol; these are then converted to sucrose by a long gluconeogenic pathway (4,12). In castor bean, the spherosomes containing the reserve triacylglycerols possess an acid lipase which is already active in the dry seed before germination (13,14). In jojoba, an alkaline lipase (wax ester hydrolase) is present in the spherosomes (wax bodies) of germinated but not dry seeds (15). In other oilseed species, lipase activities in the spherosomes of dry or germinated seeds are absent or have not been reported (5). Many studies have been conducted on the general nature and fatty acid level of soybeans, but triacylglycerol, the main component of the cotyledons, has not been investigated at the molecular level. The present study was carried out to isolate triacylglycerols from the cotyledons of soybean seedlings and to determine changes in molecular species composition and fatty acid distribution of triacylglycerols during germination.

MATERIALS AND METHODS

Germination of Seeds

Mature seeds (*Glycine Max* L) of an early variety of soybean, *Okuhara,* were harvested at Hokkaido Prefecture, Japan, 1982 and selected for uniformity based on seed weight (i.e. 285.2 to 315.6 mg). Seeds were surface sterilized with 2.5% $NaOCl_2$, soaked in water for 12 hr at 4 C and allowed to germinate on moist paper towels at 28 C in the dark (16).

Seedlings were removed from the growth chamber after 2, 4, 6, 8 and 12 days of growth, and the cotyledons were removed with a micro-spatula.

Lipid Analyses

Cotyledons were extracted 3 times with chloroform/methanol (once 1:1 and then twice 2:1, v/v) in a Waring Blender. Combined extracts of total lipids were purified as per Folch et al. (17), and then dried over anhydrous Na_2SO_4. Solvents were removed from filtrates with a rotary evaporator under nitrogen at 30-35 C. Triacylglycerols were isolated from other lipid classes by TLC on plates coated to 0.5 mm thickness with Silica Gel G. Ca. 50-60 mg of total lipids were streaked on each plate, and plates were developed with 20% diethyl ether in *n*-hexane. The appropriate band was scraped from the plate and triacylglycerols

recovered from adsorbent by extraction with 10% methanol in diethyl ether.

Triacylglycerol Composition

Triacylglycerols isolated by TLC were analyzed using GLC by the method of Matsui et al. (18), using a Shimadzu Model 7AG gas chromatograph equipped with a hydrogen flame ionization detector. A glass column (50 cm x 2.6 mm i.d.) was packed with 2% OV 101 (phenyl methyl silicone) supported on Chromosorb W HP (100/120 mesh). Helium carrier gas was used at a flow rate of 60 ml/min. The column temperature was programmed from 250 C to 340 C at 4 C/min. Triacylglycerol peaks were identified by co-chromatography with known standards. Peak areas were calculated by addition of a known weight (20 to 100 μg) of trilaurin as an internal standard using an electronic integrator (Shimadzu C-RIB). In the quantitative analysis of high molecular weight triacylglycerols, the calibration factors determined by the internal normalization technique (19) were as follows: C-36 (1.00), C-42 (1.03), C-48 (0.95), C-50 (0.87), C-52 (0.84), C-54 (0.78), C-56 (0.72) and C-58 (0.70), respectively.

Triacylglycerol Species Analysis

Molecular species analysis of total triacylglycerols was performed by silver nitrate-silica gel TLC (11). Plates were coated to 0.3 mm thickness with a slurry of 35 g of Silica Gel G and 8.8 g of silver nitrate dissolved in 70 ml of water. Freshly prepared plates were activated at 110-120 C for 2 hr, then stored before use in a desiccator in the dark. Triacylglycerol molecular species were separated by argentation TLC using 0.8% to 5.0% methanol in chloroform, depending on their degree of unsaturation (20). For quantitation of species containing the trienoic acid, linolenic acid, plates were streaked with 10-15 mg triacylglycerol and developed with 5.0% methanol in chloroform. Remaining species were separated by streaking 8-10 mg triacylglycerol on the plates and by developing these plates with 0.8-1.5% methanol in chloroform. The methanol in chloroform system was varied according to temperature and humidity conditions. Individual bands were visualized under ultraviolet light after spraying with 0.2% 2′,7′-dichlorofluorescein in ethanol. Bands were recovered from the plate by extraction with 10% methanol in diethyl ether in a Buchner funnel, followed by acidification of the adsorbent with 10% aqueous HCl in a separatory funnel and extraction with diethyl ether. Determination of relative amounts of each triacylglycerol subfraction was carried out by comparison of fatty acid methyl esters with a known amount (10 to 100 μg) of methyl pentadecanoate as an internal standard. The subfraction was converted into fatty acid methyl esters by heating it with boron trifluoride-methanol (21), and analyzed by GLC as described earlier (16). Peak areas were calibrated with standard fatty acid mixture (F & OR mixtures No. 3, Applied Science, State College, Pennsylvania) and then calculated as mole% of each fatty acid with the aid of an electronic integrator (Shimadzu C-RIB). The other GLC conditions were the same as previously described (22).

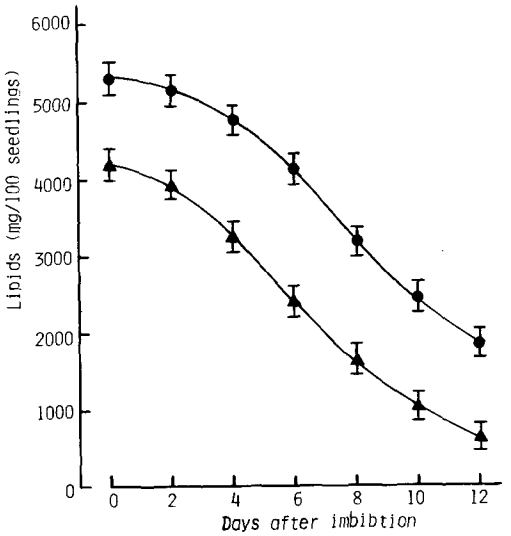

FIG. 1. Changes in the lipid contents of soybean cotyledons during seedling growth. Lipids were extracted from fresh cotyledons with chloroform/methanol (2:1 and 1:1, v/v). Triacylglycerols were separated by TLC and the amount determined by GLC, using trilaurin as an internal standard. Each bar represents mean ± SEM (n = 3). ● = total lipids; ▲ = triacylglycerols.

Lipase Hydrolysis

Hydrolysis generally followed the procedure of Mattson and Volpenhein (23). Briefly, at least 5 mg of triacylglycerol (from more than one TLC plate if necessary) was suspended in 2.0 ml of Tris buffer (pH 7.6) to which was added 35 μl of 1% aqueous solution of bile salts (Wako Junyaku Co., Ltd., Tokyo), 60 μl saturated aqueous $CaCl_2$ and 10 mg of purified pancreatic lipase (Sigma Chemical Co., St. Louis, Missouri). The reaction was carried out in a water bath at 37 C with vigorous stirring for 8 min, then the mixture was poured into diethyl ether over anhydrous Na_2SO_4 and filtered. The ether solution was concentrated to

a small volume under a stream of nitrogen. Residual 2-monoacylglycerols were separated by TLC on Silica Gel G with *n*-hexane/diethyl ether/formic acid (70:30:2, v/v/v). The appropriate band was scraped directly into an ampule and esterified by heating it with boron trifluoride-methanol (21). Fatty acid composition of the methyl esters was determined by GLC.

All the experiments in this paper have been replicated at least 3 times.

RESULTS

With continuous growth of soybean seedlings, the amounts of triacylglycerols as well as total lipids rapidly decreased, by 29.2% within 4 days, 55.0% in 8 days and 69.5% in 10 days (Fig. 1). At day 12 of seedling growth, ca. 85% of the triacylglycerols had been consumed.

Cotyledons contained even numbered triacylglycerols from C-50 to C-60 (those with contents >5.0 mg/100 seedlings) as shown in Table 1, and the initial mole% of each of these components was 3.4 for C-50, 31.7 for C-52, 63.1 for C-54, 1.4 for C-56 and 0.4 for C-58. Dominant components consisted of C-52 and C-54 triacylglycerols (1311 mg and 2619 mg/100 seedlings, respectively), whereas the other triacylglycerols were very minor components (less than ca. 140 mg/100 seedlings). With continuous growth of soybean seedlings, the amounts of the various triacylglycerols decreased substantially, by 6.0-25.9% at day 4 and by 36.5-71.9% at day 8. Decreases differed in magnitude, depending on the carbon number of the triacylglycerols. By day 12 of growth, reductions ranged from 73.6% for C-58 to 90.1% for C50.

Fourteen different molecular species were detected during germination (Table 2). For 12 days after the start of imbibition, most species containing saturated fatty acid (S_2M, SM_2, S_2D, SMD, SD_2, SMT and SDT) decreased drastically to less than 10% of the original content. Two species containing linolenic acid (MDT and D_2T) also decreased by 0.2% and 11.5%, respectively, by 12 days. However, the other species represented from 42.4-69.2% of their original (day 0) values by 8 days to 23.2-36.2% by 12 days.

In order to obtain information on isomer composition, the distribution of fatty acids in the primary (α) and secondary (β) positions of the triacylglycerol were determined via lipase hydrolysis. Very little of the saturated fatty acids were in the 2-position in the triacylglycerols of the cotyledons of soybean seedlings (Table 3). A third of the 18:1 remained in the 2-position after imbibition with no appreciable

TABLE 1

Changes in the Triacylglycerol Content of Germinating Soybean Cotyledons[a]

Days after imbibition	Carbon number[b]											
	50		52		54		56		58			
	mg[c]	Relative content (%)	mg	Relative content (%)	mg	Relative content (%)	mg	Relative content (%)	mg	Relative content (%)		
0	141.0	100	1311	100	2619	100	58.1	100	20.8	100		
4	104.5[d]	74.1	999.3[d]	76.2	2103[d]	80.3	53.7	92.5	19.5	94.0		
8	39.6[e]	28.1	474.8[e]	36.2	1087[e]	41.5	33.0[d]	56.8	13.2[d]	63.5		
12	13.9[f]	9.9	160.6[f]	12.3	412.7[f]	15.8	13.8[e]	23.8	5.5[e]	26.4		

[a]Each value is an average of 3 determinations.
[b]Length of total acyl chains present in a triacylglycerol.
[c]Expressed as mg per 100 seedlings. Minor components have been omitted (<5.0 mg).
[d,e,f]Values in a column without the same superscript are significantly different from those immediately after imbibition ($^{d}p < 0.05$, $^{e}p < 0.02$, $^{f}p < 0.01$).

TABLE 2

The Residual Molecular Species of Triacylglycerols from Germinating Soybean Cotyledons[a,b]

Triacylglycerol species[c]	No. of double bonds	% total at day 0	Days after imbibition 0	4	8	12
S_2M	1	4.8	100	62.2[d]	14.9[e]	2.4[f]
SM_2	2	3.2	100	62.3[d]	16.2[e]	0.9[f]
S_2D	2	5.2	100	65.8[d]	27.1[e]	4.8[f]
M_3	3	6.9	100	85.4[d]	45.5[e]	25.2[f]
SMD	3	10.3	100	63.4[d]	25.9[e]	8.3[f]
M_2D	4	7.7	100	95.9	42.4[d]	23.4[e]
SD_2	4	12.5	100	70.9[d]	28.9[e]	8.1[f]
SMT	4	2.5	100	72.5[d]	30.3[e]	7.3[f]
MD_2	5	9.6	100	94.7	69.2[d]	36.2[e]
M_2T	5	2.8	100	83.7[d]	42.6[e]	27.7[e]
SDT	5	4.8	100	73.2[d]	32.5[e]	8.5[f]
D_3	6	15.9	100	95.1	51.4[d]	23.2[e]
MDT	6	5.6	100	73.1[d]	30.5[e]	9.2[f]
D_2T	7	8.2	100	74.9[d]	35.9[e]	11.5[f]

[a]Each value is an average of 3 determinations.

[b]The residual amounts (relative percentage) were calculated in comparison with a known amount of methyl pentadecanoate as an internal standard, and each value at day 0 was normalized to 100.

[c]Saturated fatty acids (S) consisting of palmitic (16:0) and stearic (18:0) acids. Unsaturated fatty acids, oleic (18:1), linoleic (18:2), and linolenic (18:3), are denoted as monoene (M), diene (D) and triene (T), respectively.

[d,e,f]Values without the same superscript are significantly different from those immediately after imbibition ($^dp < 0.05$, $^ep < 0.02$, $^fp < 0.01$).

TABLE 3

Per Cent Distribution of Fatty Acids Esterified in the 2-Position of Total Triacylglycerols Isolated from Germinating Soybean Cotyledons as Determined by Lipase Hydrolysis[a,b]

Fatty acid	Days after imbibition 0	4	8	12
16:0	0.8	1.1	2.4[c]	3.0[d]
18:0	1.2	1.8	2.3[c]	2.3[c]
18:1	33.3	34.7	33.3	33.7
18:2	44.0	44.8	48.5[c]	52.7[c]
18:3	26.3	25.9	31.3[c]	48.1[d]

[a]Mole% distribution of fatty acyl chains esterified in the 2-position. Formular example: (mole% 18:2 in 2-position)/(3 × mole% 18:2 Triacylglycerol) × 100 = percentage 18:2 in triacylglycerol that is located in 2-position.

[b]Each value is an average of 3 determinations.

[c,d]Values are significantly different from those immediately after imbibition ($^cp < 0.05$, $^dp < 0.01$).

changes. With continuous growth of soybean seedlings, the percentage of 18:2 and 18:3 gradually increased in the 2-position.

Selected data for individual triacylglycerol species isolated from total lipids in the cotyledons of soybean seedlings are shown in Table 4. This detailed information indicates the existence of specific distribution patterns for the individual fatty acids; oleic acid was highly concentrated in the 2-position (S_2M and SM_2). However, when the diene was present, it dominated in the 2-position at the expense of the monoene or triene. The saturates were consistently low in this position.

DISCUSSION

Data presented here show that germination and seedling growth in soybeans are accompanied by a substantial decline in the triacylglycerols. The extent of this decline is inversely proportional to the length of the acyl chain present in acylglycerols. With continuous growth of soybean seedlings, the amounts of C-58 triacylglycerol decreased. Large reductions were evident in the amount of triacylglycerol of shorter acyl chain lengths in the cotyledons. This indicates that the triacylglycerols of shorter acyl chain length may be preferentially metabolized in cotyledons of soybean seedlings relative to longer triacylglycerols. In addition, there are greater reductions in molecular species of triacylglycerols containing saturated fatty acids throughout germination of soybean seeds. Three of these species (S_2M, SM_2 and S_2D) became very minor components by 12 days. However, residual amounts (relative percent-

TABLE 4

Per Cent Distribution of Fatty Acids Esterified in the 2-Position of Individual Triacylglycerol Species Isolated from Germinating Soybean Cotyledons[a,b]

Days after imbibition	Triacylglycerol species[c]	No. of double bonds	Fatty acid				
			16:0	18:0	18:1	18:2	18:3
0	S_2M	1	11.5	4.2	84.3		
	SM_2	2	3.4	–	96.6		
	S_2D	2	7.6	9.3		83.1	
	SMD	3	2.7	–	28.5	68.8	
	MD_2	5			12.9	87.1	
	D_2T	7				82.6	17.4
8	S_2M	1	10.8	4.7	84.5		
	SM_2	2	3.8	–	96.2		
	S_2D	2	7.9	10.1		82.0	
	SMD	3	3.2	–	27.9	68.9	
	MD_2	5			13.5	86.5	
	D_2T	7				82.1	17.9
12	S_2M	1	9.8	4.6	85.6		
	SM_2	2	4.2	–	95.8		
	S_2D	2	8.2	11.3		80.5	
	SMD	3	3.4	–	26.8	69.8	
	MD_2	5			15.2	84.8	
	D_2T	7				80.5	19.5

[a]Each value is given as mole% of total fatty acyl chains in the 2-position and represents an average of 3 determinations. Minor components have been omitted (<0.09).

[b]Values are not significantly different from those immediately after imbibition ($p < 0.05$).

[c]Abbreviations: see Table 2.

ages) of the triacylglycerol molecular species consisting of only unsaturated fatty acids (M_3, M_2D, MD_2, M_2T, D_3 and D_2T) were generally higher in comparison to those of other species containing saturated fatty acids. Germination and seedling growth of seeds are accompanied by the mobilization of reserves present in storage tissues, cotyledons, endosperm, etc. (24). On the basis of these data, it is considered that some triacylglycerol molecular species (S_2M, SM_2 and S_2D) are preferentially mobilized to support the growth of the storage tissues and the embryonic axis.

In soybean oil, saturated fatty acids (16:0 and 18:0) are dominant components in the 1,3-positions, but 18:3 is distributed in all positions at random (1). However, in soybean seeds, patterns of triacylglycerol molecular species and their fatty acid distributions changed during germination and seedling growth. It is likely that those triacylglycerols which contain saturated fatty acids are the first to be completely hydrolyzed. This indicates that the mechanism of initial triacylglycerol hydrolysis may be different in various molecular species.

It previously was thought that seed lipases were generally non-specific and could hydrolyze a wide variety of triacylglycerols. In contrast, Lin et al. (25,26) found that glyoxysomal lipase which catalyzes the initial hydrolysis of storage triacylglycerols was active toward trilinolein, dilinolein and monolinolein, but not active toward triolein, tristearin and tripalmitin. At present, however, it is not possible to predict whether or not a specific lipase functions in hydrolysis in oil seeds. Unlike the simple triacylglycerols acted on by glyoxysomal lipase, natural triacylglycerols of oil seeds are composed of a complex mixture of molecular species. These species consist of fatty acids of different lengths, and there are double bonds on the fatty acyl chains present in the triacylglycerols.

Although the triacylglycerols of the cotyledons serve mainly as reserve energy sources (27), it would be of interest to pursue studies on the biological properties of the molecular species of triacylglycerols residing during germination in regards to their fatty acid distributions in the *sn*-1, 2 and 3-positions.

REFERENCES

1. Brockerhoff, H., and Yurkowski, M. (1966) J. Lipid Res. 7, 62-64.
2. Wada, S., Koizumi, C., and Nonaka, J. (1977)

Yukagaku 26, 95-99.
3. Petersson, B., Podlaha, O., and Töregård, B. (1981) J. Am. Oil Chem. Soc. 58, 1005-1009.
4. Hutto, D., and Stumpf, P.K. (1969) Plant Physiol. 44, 508-516.
5. Huang, A.H.C., and Moreau, R.A.. (1978) Planta 141, 111-116.
6. Weber, E.J. (1973) Lipids 8, 295-302.
7. Wilson, R.F., and Rinne, R.W. (1978) Plant Physiol. 61, 830-833.
8. Ichihara, K.-I., and Noda, M. (1980) Phytochemistry 19, 49-54.
9. Gurr, M.I., Blades, J., and Appleby, R.S. (1972) Eur. J. Biochem. 29, 362-368.
10. Gurr, M.I., Blades, J., Appleby, R.S., Smith, C.G., Robinson, M.P., and Nichols, B.W. (1974) Eur. J. Biochem. 43, 281-290.
11. De la Roche, I.A., Weber, E.J., and Alexander, D.E. (1971) Lipids 6, 537-540.
12. Huang, A.H.C. (1975) Plant Physiol. 55, 555-558.
13. Ory, R.L., Yatsu, L.Y., and Kircher, H.W. (1968) Arch. Biochem. Biophys. 264, 255-264.
14. Muto, S., and Beevers, H. (1974) Plant Physiol. 54, 23-28.
15. Huang, A.H.C., Moreau, R.A., and Liu, K.D.F. (1978) Plant Physiol. 61, 339-341.
16. Yoshida, H., and Kajimoto, G. (1977) Agric. Biol. Chem. 41, 1857-1864.
17. Folch, J., Lees, M., and Sloane Stanley, G.H. (1957) J. Biol. Chem. 226, 497-509.
18. Matsui, M., Watanabe, T., and Ikekawa, N. (1973) Nippon Suisan Gakkaishi 39, 367-373.
19. Litchfield, C., Harlow, R.D., and Reiser, R. (1965) J. Am. Oil Chem. Soc. 42, 849-857.
20. Blank, M.L., Verdino, B., and Privett, O.B. (1965) J. Am. Oil Chem. Soc. 42, 87-90.
21. Morrison, W.R., and Smith, L.M. (1964) J. Lipid Res. 5, 600-608.
22. Yoshida, H., and Kajimoto, G. (1980) Agric. Biol. Chem. 44, 183-191.
23. Mattson, F.H., and Volpenhein, R.A. (1961) J. Lipid Res. 2, 58-62.
24. Baumgartner, B., Tokuyasu, K.T., and Chrispeels, M.J. (1978) J. Cell Biol. 79, 10-19.
25. Lin, K.D.F., Moreau, R.A., and Huang, A.H.C. (1982) Plant Physiol. 70, 108-112.
26. Lin, K.D.F., Wimer, L.T., and Huang, A.H.C. (1983) Plant Physiol. 73, 460-463.
27. Wanner, G., and Theimer, R.R. (1978) Planta 140, 163-169.

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Homeostatic Control of Membrane Fatty Acid Composition in the Rat After Dietary Lipid Treatment

R.A. GIBSON,*[a] E.J. MCMURCHIE,[b] J.S. CHARNOCK[b] and G.M. KNEEBONE,[a]
[a]Department of Pediatrics, Flinders Medical Center, Bedford Park, South Australia 5042, Australia, and [b]CSIRO, Division of Human Nutrition, Glenthorne Laboratories, Majors Road, O'Halloran Hill, South Australia 5158, Australia

ABSTRACT

Diets in which both the lipid content and composition (polyunsaturated to saturated fatty acid ratio) were varied were fed to rats for 20 weeks, and the effects on the tissue lipid profiles were determined. The fatty acid profile of the plasma lipids, and the phospholipid fatty acids of the mitochondrial and microsomal fractions of liver, heart, kidney and brain, as well as erythrocyte membranes were determined. Despite large differences in the level and type of lipid present in the experimental diets and in the proportion of saturated fatty acids in the plasma lipids in response to the various diets, there was little effect on the proportion of saturated to unsaturated fatty acids in the phospholipids of the various membranes examined. The major effect of altering the dietary level of polyunsaturated to saturated fatty acids was on the ratio of the $\omega6/\omega3$ series of unsaturated fatty acids in the membrane lipids. This change occurred in all tissues except the brain, in which only a small response to altered dietary lipid intake was observed. The $\omega6/\omega3$ ratio was elevated upon feeding a diet rich in $\omega6$ polyunsaturated fatty acids, but decreased when a diet rich in saturated fatty acids was fed. The failure to significantly alter membrane lipid saturation/unsaturation in the tissues examined would suggest that a homeostatic mechanism is operative in biological membranes and may act to buffer membranes from the effects of changes in the nature of the dietary lipid intake.
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INTRODUCTION

Dietary lipids are known to influence the fatty acid composition of tissue lipids (1-3) including those of heart (4), brain (5), liver (6) and blood (7-9). Most studies have concentrated on the dietary-induced changes in the fatty acid distribution of either total lipids or the individual phospholipids from unfractionated tissues and organs (10,11). However, recent studies have focused on dietary-induced changes in specific subcellular membranes (12-15). These changes are now being recognized as functionally important as the specific role of subcellular membrane lipids in modulating membrane function becomes more clear (16-18). This is particularly so with regard to the effects of dietary lipids on the physical properties of membrane lipids and the effect they have on the functioning of the various membrane enzymes associated with many physiological processes (19-22).

As part of a larger survey on the effects of dietary lipids on the physical and biochemical properties of cellular membranes and associated processes, we have examined the changes in subcellular membrane lipid composition following changes in the nature of the dietary lipid intake. Of particular interest have been the effects of diets of widely differing lipid composition on both the level of membrane lipid saturated/unsaturated fatty acids and the nature of the unsaturated fatty acids present, particularly with regard to the relationship between the $\omega9$ (oleate), $\omega6$ (linoleate) and $\omega3$ (linolenate) series of unsaturated fatty acids. In addition to the biophysical effects of changing membrane lipid composition, the established role of these fatty acid families in prostanoid metabolism (23-25) supports the relevance of such a study.

We show that in the rat, subcellular membrane phospholipid fatty acid composition can be altered by dietary lipid treatment. However, for any one organ the change is predominantly in the type of membrane unsaturated fatty acids present rather than in differences in the ratio of the saturated to unsaturated fatty acids. The subcellular membranes from tissues that do respond significantly to changes in the dietary lipid intake appear to exhibit considerable homeostasis in that a constant level is maintained for both the proportion of lipid unsaturation and the value of the unsaturation index, despite wide differences in the values of these two parameters in the various experimental diets used.

MATERIALS AND METHODS

Male rats (Hooded Wistar), weighing be-

*To whom correspondence should be addressed.

tween 242 and 315 g (mean 277 g) were maintained on the various lipid-supplemented diets described below for 20 weeks. At the time of sacrifice the body weights were, REF. group 438 ± 7.5 g (mean ±SE; n = 18), SAT. group 497 ± 7.8 g (n = 18) and UNSAT. group 495 ± 7.2 g (n = 18). Rats were killed by decapitation and the tissues removed immediately for preparation of subcellular membrane fractions.

Rat Diets

The basic rat ration was composed of 52% wheat starch, 18% milling byproducts (including starch, fiber and crude protein), 7% meat and bone meal, 6% cottonseed meal, 6% fishmeal, 5% soybean meal, 1% brewers yeast, 3% salt mix and 2% vitamin mix. Vitamins and minerals were added to the mixture in the following amounts per kilogram: choline, 525 mg; vitamin E, 36 mg; vitamin K_3, 18 mg; thiamine, 58 μg; vitamin A, 22,000 I.U.; pantothenic acid, 16 mg; niacin, 18 mg; vitamin B_{12}, 0.12 mg; vitamin D_3, 4,000 I.U.; biotin, 0.12 mg; riboflavin, 5 mg; pyridoxine, 5 mg; folic acid, 2 mg; magnesium, 415 mg; iron, 66 mg; manganese, 114 mg; zinc, 64 mg; copper, 5 mg; molybedenum, 1 mg; iodine, 2 mg, and cobalt, 0.4 mg. The fat content of the diet was either 4% or 16%, depending on the nature of the experimental diet. The fat was either a basal mixture of vegetable oils as used in normal rat ration (Australasian Feed Services, Australia) for the reference (REF.) diet or 4% basal fat plus 12% sheep perirenal fat for the saturated fatty acid (SAT.) diet or 4% basal fat plus 12% sunflower seed oil (Nuttelex Pty. Ltd., Australia) for the unsaturated fatty acid (UNSAT.) diet. The fat was added to the commercial mixture at the time of pelleting. Fatty acid analysis of the diets is shown in Table 1.

TABLE 1

Fatty Acid Composition of Normal and Lipid Supplemented Rat Diets

Fatty acid[1] (%; w/w)	REF.[2]	SAT.[2]	UNSAT.[2]
14:0	1.5	2.5	0.3
16:0	18.6	21.1	8.2
16:1	3.1	2.2	0.7
17:0	0.6	1.9	0.2
18:0	6.7	29.1	4.9
18:1	22.6	31.5	22.6
18:2ω6	33.2	8.6	60.3
18:3ω3	3.1	1.3	0.9
20:1	3.4	0.8	0.7
22:1/20:5	4.7	0.6	0.7
22:6ω3	2.4	0.3	0.4
SAT.	27.4	54.6	13.6
UNSAT.	72.6	45.4	86.4
UNSAT./SAT.	2.6	0.8	6.4

[1] Fatty acids are designated by the number of carbon atoms followed by the number of double bonds. The position of the first double bond relative to the methyl or omega (ω) end of the molecule also is indicated.

[2] REF. (Reference, standard laboratory diet); SAT. (sheep kidney fat supplemented diet); UNSAT. (sunflower seed oil supplemented diet). Trace amounts (less than 0.3%) also were detected for the fatty acids 20:0 and 22:0.

Combustion calorimetry of the REF., SAT. and UNSAT. diets gave energy values of 17.1, 20.1 and 20.0 KJ/g respectively. Animals fed the SAT. and UNSAT. diets consumed about 20 g of food per day compared with approximately 25 g of food per day for the animals in the REF. group. All diets were supplied ad libitum.

Blood and Plasma Collection

Immediately after the animals were killed, blood was collected and centrifuged to separate plasma and red blood cells. The red blood cells were washed 3 times by centrifugation in phosphate buffered saline.

Liver, Kidney and Brain Mitochondrial Fractions

For preparation of mitochondria from the above tissues, about 4 g from one liver, both kidneys or all the brain tissue from one animal were chopped and rinsed in ice-cold medium containing 250 mM sucrose, 2 mM Hepes (4-(2-hydroxy-ethyl)-1 piperazine ethane-sulphonic acid), 0.5 mM EGTA and 0.05% (w/w) delipidated bovine serum albumin (BSA), pH 7.4 and then homogenized in 40 ml of the above medium using a Polytron tissue homogenizer (Kinematica, GmbH, Switzerland) at setting 3.5 for 2 bursts each of 6 seconds. The homogenate was filtered through cheesecloth and centrifuged at 500 g for 12 min and the supernatant saved. The resulting pellet was resuspended in medium to the original volume and recentrifuged at 500 g for 12 min. The supernatant from this and the previous centrifugation were combined and centrifuged at 6000 g for 15 min. The resulting supernatant was saved for preparation of the microsomal fraction described below. The mitochondrial pellet from the above centrifugation was washed twice by centrifugation in the above medium at 6000 g for 15 min. and finally resuspended in this medium.

Heart Mitochondrial Fraction

Ventricular tissue from one animal was chopped and rinsed in ice-cold medium contain-

ing 100 mM 50 mM Mops (4-Morpholinepropanesulphonic acid), 2 mM EGTA and 0.2% (w/v) delipidated BSA, pH 7.2 and then homogenized in 40 ml of the above medium in a manner identical to that described for the preparation of mitochondria from the other tissues. The procedure for the subsequent isolation of heart mitochondria by differential centrifugation was as described for the other tissues, except that the first mitochondrial pellet was resuspended in the above medium without BSA. After washing the mitochondrial pellet by centrifugation, the pellet was then finally resuspended in the buffer without BSA. The supernatant resulting from the first centrifugation at 6000 g for 15 min was saved for the preparation of the heart microsomal fraction.

Liver, Kidney, Brain and Heart Microsomal Fractions

The supernatants from the first 6000 g centrifugation described above were centrifuged at 110,000 g for 60 min and the pellet collected and referred to as the microsomal membrane fraction.

Lipid Analysis

Prior to lipid analysis, the mitochondrial and microsomal membrane preparations from the liver, kidney, brain and heart were diluted in 50 volumes of 20 mM Tris, 2 mM EDTA, pH 7.2 and centrifuged at 250,000 g for 60 min to remove sucrose or KCl. The resulting membrane pellets were resuspended in glass distilled water, prior to extraction of lipids using a modification of a method developed for erythrocyte membranes (26). Briefly, to one volume of plasma, washed red blood cells or washed mitochondrial or microsomal membrane preparations, 4 volumes of boiling 2-propanol were added and the mixture was boiled for 30 seconds. After cooling, 8 volumes of chloroform containing the antioxidant butylated hydroxyanisole (0.1% of the estimated lipid weight) were added. Following the addition of 1 volume of glass distilled water, the samples were shaken, centrifuged and the organic phase collected. After re-extracting the aqueous phase with a further 4 volumes of chloroform, the organic phases were combined and dried using anhydrous sodium sulphate. For all but the plasma lipids, phospholipids were separated from the total extracted lipids by thin-layer chromatography (TLC) on silica gel H plates developed in petroleum ether/diethyl ether/acetic acid, (90:15:1). The phospholipids remaining at the origin were removed and methylated in 1% (v/v) H_2SO_4 in methanol by heating at 70 C for 3 hr. For plasma, the fatty acids of the total lipids were methylated. For analysis of the fatty acids present in the various dietary lipid supplements, the diet pellets were extracted and fatty acid analysis was performed on the total lipid extract.

Gas Chromatography

Fatty acid methyl esters were analyzed using a Hewlett-Packard 5850A gas chromatograph fitted with dual FID detectors. Operating conditions were as follows: the FID was set at 300 C, the injection port at 200 C and the oven temperature programmed from 125 to 225 C at a heating rate of 4 C/min. Glass columns were packed with 5% SP2310 on chromasorb WAW (Supelco Inc. Bellefonte, Pennsylvania). This packing allows for the separation of up to 4 double bonds of one homologous series of fatty acids before encountering the parent saturate of the next series, thus resulting in minimal overlap of the major membrane phospholipid fatty acids. For example, 18:3ω3 was clearly separated from 20:0 and 20:4ω6 emerged before 22:0. Two overlaps which did occur in this system were 20:3ω9 with 20:2ω6, and also 20:5ω3 with 22:1, but as none of these compounds were present in large amounts in the samples examined, no attempt was made to estimate the amounts of the individual fatty acids by rechromatography on different packings.

RESULTS

The fatty acid composition of the various lipid-supplemented diets is shown in Table 1. Addition of sheep kidney perirenal fat (SAT. diet) to the normal or REF. diet increased the proportion of myristic (14:0), palmitic (16:0), stearic (18:0) and oleic (18:1ω9) acids. Sunflower seed oil (UNSAT. diet) increased lipid unsaturation to about 86%, mainly by elevating the proportion of linoleic acid (18:2ω6). The total caloric energy derived from linoleic acid represented 2.5, 12.4 and 23.9% of the dietary energy intake for the SAT., REF. and UNSAT. diets, respectively. These levels are all above the minimum requirement for linoleic acid of 1.3% of the total dietary energy intake in male rats (27). All diets also contained 0.9% or greater linolenic acid (18:3ω3), but no diet contained any measurable quantity of arachidonic (20:4ω6) or eicosapentaenoic (20:5ω3) acids. Only trace amounts of docosahexaenoic acid (22:6ω3) were present in the SAT. and UNSAT. diets.

The fatty acid composition of the plasma lipids and the red blood cell (erythrocyte) phospholipids, as well as the phospholipids from the various subcellular membrane fractions of the tissues examined, are shown in Tables 2 to 6. A summary of all these tables in

TABLE 2

Major Fatty Acids of Plasma Lipids and Red Blood Cell Phospholipids from Rats Fed Lipid-Supplemented Diets for 20 Weeks

Fatty acid (%; w/w)	Plasma			Red blood cells		
	REF.	SAT.	UNSAT.	REF.	SAT.	UNSAT.
14:0	0.6 ± 0.04	0.8 ± 0.07	0.2 ± 0.02	0.3 ± 0.03	0.2 ± 0.01	0.2 ± 0.01
DMA 16:0	0.1 ± 0.01	0.1 ± 0.01	0.1 ± 0.01	3.3 ± 0.04	2.4 ± 0.17	2.3 ± 0.10
16:0	18.7 ± 0.70	15.5 ± 0.72	14.5 ± 1.00	28.4 ± 0.14	23.6 ± 0.52	25.3 ± 0.58
16:1	4.0 ± 0.18	2.9 ± 0.09	0.3 ± 0.28	–	–	–
17:0	0.8 ± 0.04	1.1 ± 0.07	0.5 ± 0.07	1.2 ± 0.05	1.5 ± 0.06	1.1 ± 0.04
DMA 18:0	0.4 ± 0.02	0.3 ± 0.04	0.1 ± 0.03	3.1 ± 0.13	3.1 ± 0.17	2.6 ± 0.14
DMA 18:1	–	–	–	1.2 ± 0.09	1.3 ± 0.05	1.2 ± 0.06
18:0	11.1 ± 0.55	13.8 ± 0.42	13.3 ± 1.13	13.1 ± 0.22	17.0 ± 0.29	17.1 ± 0.35
18:1	14.9 ± 0.43	25.8 ± 1.33	13.8 ± 0.68	9.3 ± 0.14	11.3 ± 0.20	8.1 ± 0.13
18:2ω6	19.6 ± 0.57	15.1 ± 0.17	30.1 ± 0.72	8.7 ± 0.08	9.7 ± 0.30	11.4 ± 0.32
18:3ω6	0.3 ± 0.20	0.2 ± 0.16	0.3 ± 0.27	–	–	–
18:3ω3	0.9 ± 0.07	0.9 ± 0.05	0.5 ± 0.15	–	–	–
20:0	0.4 ± 0.04	0.8 ± 0.04	0.5 ± 0.02	0.2 ± 0.01	0.2 ± 0.02	0.3 ± 0.02
20:1	1.1 ± 0.08	1.0 ± 0.04	0.9 ± 0.04	0.3 ± 0.02	0.3 ± 0.02	0.4 ± 0.03
20:2ω6+20:3ω9	0.4 ± 0.03	0.4 ± 0.03	0.5 ± 0.04	0.2 ± 0.02	0.3 ± 0.04	0.5 ± 0.02
20:3ω6	0.7 ± 0.05	0.6 ± 0.08	0.4 ± 0.05	0.1 ± 0.03	0.5 ± 0.03	0.3 ± 0.01
20:4ω6	20.1 ± 1.11	14.3 ± 1.13	20.7 ± 1.55	21.4 ± 0.30	20.1 ± 0.56	21.9 ± 0.52
22:0	–	–	–	–	–	–
22:1+20:5ω3	1.3 ± 0.16	1.1 ± 0.16	0.6 ± 0.19	0.7 ± 0.01	0.9 ± 0.03	0.1 ± 0.01
22:4ω6	0.3 ± 0.05	0.2 ± 0.05	0.5 ± 0.06	1.1 ± 0.4	0.7 ± 0.05	1.9 ± 0.07
24:0	0.4 ± 0.06	0.3 ± 0.03	0.7 ± 0.05	1.4 ± 0.09	1.1 ± 0.04	1.7 ± 0.11
24:1+22:5ω6	–	–	–	0.6 ± 0.05	0.7 ± 0.04	0.6 ± 0.07
22:5ω3	0.7 ± 0.08	0.3 ± 0.04	0.1 ± 0.02	1.4 ± 0.04	1.4 ± 0.07	0.5 ± 0.02
22:6ω3	3.0 ± 0.22	3.4 ± 1.05	1.3 ± 0.10	3.7 ± 0.05	3.8 ± 0.19	2.6 ± 0.20

Fatty acids are designated as described in Table 1. Data are presented as the mean relative percentage ±SEM for n = 6 animals in each dietary group. (–) = not detected or present at less than 0.1%.

TABLE 3

Major Fatty Acids of the Phospholipids of the Mitochondrial and Microsomal Membrane Fractions from the Liver of Rats Fed Lipid-Supplemented Diets for 20 Weeks

Fatty acid (%; w/w)	Mitochondria			Microsomes		
	REF.	SAT.	UNSAT.	REF.	SAT.	UNSAT.
14:0	0.2 ± 0	0.1 ± 0	0.1 ± 0	0.2 ± 0	0.1 ± 0	0.1 ± 0
DMA 16:0	0.1 ± 0	0.1 ± 0	0.1 ± 0	0.1 ± 0	0.1 ± 0	0.1 ± 0
16:0	15.1 ± 0.2	11.1 ± 0.3	11.7 ± 0.1	16.4 ± 0.2	11.6 ± 0.2	12.8 ± 0.3
16:1	2.6 ± 0.1	1.3 ± 0	0.1 ± 0	2.5 ± 0.1	1.3 ± 0	0.01 ± 0
17:0	0.7 ± 0.1	0.8 ± 0	0.7 ± 0	0.9 ± 0	0.9 ± 0	0.7 ± 0
DMA 18:0	0.5 ± 0	0.3 ± 0	0.3 ± 0	0.5 ± 0	0.4 ± 0	0.4 ± 0
DMA 18:1	–	–	–	–	–	–
18:0	18.4 ± 0.2	23.1 ± 0.8	21.3 ± 0.3	21.1 ± 0.5	23.9 ± 0.4	24.9 ± 0.3
18:1	9.5 ± 0.3	11.0 ± 0.5	8.3 ± 0.2	9.6 ± 0.2	11.1 ± 0.4	7.3 ± 0.1
18:2ω6	15.0 ± 0.3	12.4 ± 0.7	14.0 ± 0.7	12.1 ± 0.1	10.7 ± 0.4	10.7 ± 0.2
18:3ω6	0.3 ± 0.3	0.4 ± 0.2	–	0.3 ± 0.2	0.4 ± 0.3	0.1 ± 0.1
18:3ω3	–	–	–	–	–	–
20:0	0.2 ± 0	0.2 ± 0	0.3 ± 0	0.2 ± 0	0.4 ± 0	0.3 ± 0
20:1	0.5 ± 0.1	0.4 ± 0	0.7 ± 0	0.4 ± 0	0.6 ± 0.1	0.7 ± 0
20:2ω6+20:3ω9	0.4 ± 0	0.4 ± 0	1.2 ± 0.1	0.4 ± 0	0.6 ± 0	0.9 ± 0
20:3ω6	1.2 ± 0.1	1.1 ± 0.1	0.5 ± 0	1.3 ± 0.1	1.3 ± 0.1	0.5 ± 0
20:4ω6	24.5 ± 0.4	25.8 ± 1.1	31.3 ± 0.5	24.6 ± 0.4	25.2 ± 0.5	32.1 ± 0.3
22:0	–	–	–	–	–	–
22:1+20:5ω3	1.4 ± 0	0.8 ± 0.2	–	1.2 ± 0.1	1.4 ± 0.1	0.2 ± 0.2
22:4ω6	0.2 ± 0	0.2 ± 0	0.5 ± 0	0.3 ± 0	0.2 ± 0	0.5 ± 0
24:0	0.5 ± 0	1.5 ± 0.1	1.5 ± 0.1	0.6 ± 0.1	0.4 ± 0	1.3 ± 0.1
24:1+22:5ω6	0.1 ± 0	–	–	0.1 ± 0	0.1 ± 0	0.1 ± 0
22:5ω3	0.5 ± 0	0.3 ± 0	0.3 ± 0	0.4 ± 0	0.4 ± 0	0.2 ± 0
22:6ω3	8.2 ± 0.3	9.2 ± 0.2	7.4 ± 0.2	6.7 ± 0.1	8.3 ± 0.2	6.0 ± 0.2

Data are as described in Table 2.

TABLE 4

Major Fatty Acids of the Phospholipids of the Mitochondrial and Microsomal Fraction from the Heart of Rats Fed Lipid-Supplemented Diets for 20 Weeks

Fatty acid (%; w/w)	Mitochondria			Microsomes		
	REF.	SAT.	UNSAT.	REF.	SAT.	UNSAT.
14:0	0.1 ± 0	0.1 ± 0	0.1 ± 0	0.1 ± 0.1	0.1 ± 0	0.1 ± 0.1
DMA 16:0	2.3 ± 0.1	1.9 ± 0.1	1.9 ± 0.1	2.6 ± 0.2	2.2 ± 0.2	2.0 ± 0.2
16:0	9.3 ± 0.2	7.9 ± 0.2	6.6 ± 0.2	9.9 ± 0.8	8.8 ± 0.5	8.3 ± 0.8
16:1	0.9 ± 0.2	1.6 ± 1.2	0.2 ± 0.2	–	–	–
17:0	0.6 ± 0	0.6 ± 0	0.3 ± 0.1	0.3 ± 0.1	0.4 ± 0	0.3 ± 0.1
DMA 18:0	1.1 ± 0	2.0 ± 0.1	1.4 ± 0.2	1.7 ± 0.1	2.3 ± 0.3	1.7 ± 0.1
DMA 18:1	0.1 ± 0.1	0.5 ± 0.1	0.7 ± 0.1	0.3 ± 0.1	0.6 ± 0.1	0.6 ± 0.1
18:0	19.9 ± 0.4	21.1 ± 0.2	23.3 ± 0.3	20.6 ± 1.0	21.1 ± 0.3	21.8 ± 0.7
18:1	9.0 ± 0.1	9.2 ± 0.1	7.2 ± 0.3	12.2 ± 0.7	11.4 ± 0.2	10.0 ± 0.5
18:2ω6	21.9 ± 0.9	15.1 ± 0.5	17.7 ± 0.5	16.9 ± 0.6	13.4 ± 0.6	16.0 ± 0.9
18:3ω6	–	–	–	–	–	–
18:3ω3	–	–	–	–	–	–
20:0	0.2 ± 0	0.2 ± 0	0.2 ± 0	0.2 ± 0	0.1 ± 0	0.2 ± 0
20:1	0.4 ± 0	0.3 ± 0.1	0.3 ± 0.1	0.8 ± 0.2	0.4 ± 0	0.5 ± 0.1
20:2ω6+20:3ω9	0.2 ± 0	0.2 ± 0	0.2 ± 0	0.3 ± 0	0.2 ± 0	0.4 ± 0
20:3ω6	0.3 ± 0	0.4 ± 0	0.3 ± 0	0.6 ± 0.1	0.5 ± 0	0.5 ± 0.1
20:4ω6	15.9 ± 0.3	18.6 ± 0.3	21.8 ± 0.3	16.4 ± 1.1	19.2 ± 0.7	21.1 ± 1.0
22:0	–	–	–	–	–	–
22:1+20:5ω3	0.4 ± 0.1	0.5 ± 0.1	0.2 ± 0.2	0.6 ± 0.3	0.6 ± 0.2	0.7 ± 0.3
22:4ω6	0.4 ± 0	0.3 ± 0	1.6 ± 0	1.4 ± 0.2	0.8 ± 0	1.9 ± 0.3
24:0	0.6 ± 0	0.4 ± 0	3.5 ± 0.1	1.3 ± 0.3	1.0 ± 0.2	3.0 ± 0.5
24:1+22:5ω6	–	–	–	–	–	–
22:5ω3	1.1 ± 0	1.4 ± 0	0.8 ± 0	1.2 ± 0.1	1.6 ± 0.1	0.9 ± 0.1
22:6ω3	15.2 ± 0.4	17.3 ± 0.3	11.5 ± 0.3	12.7 ± 0.9	15.4 ± 0.3	9.9 ± 0.6

Data are as described in Table 2.

TABLE 5

Major Fatty Acids of the Phospholipids of the Mitochondrial and Microsomal Membrane Fractions from the Kidney of Rats Fed Lipid-Supplemented Diets for 20 Weeks

Fatty acid (%; w/w)	Mitochondria			Microsomes		
	REF.	SAT.	UNSAT.	REF.	SAT.	UNSAT.
14:0	0.1 ± 0	0.1 ± 0	0.1 ± 0	0.2 ± 0	0.1 ± 0	0.1 ± 0
DMA 16:0	1.8 ± 0.1	1.3 ± 0.1	1.3 ± 0	2.8 ± 0.1	2.0 ± 0	2.4 ± 0.2
16:0	13.6 ± 0.2	13.6 ± 0.2	13.3 ± 0.3	19.2 ± 0.3	19.7 ± 0.4	19.1 ± 1.1
16:1	1.8 ± 0.1	2.0 ± 0	0.1 ± 0	–	–	–
17:0	0.5 ± 0.1	0.5 ± 0	0.5 ± 0	0.6 ± 0	0.8 ± 0	0.6 ± 0.1
DMA 18:0	0.9 ± 0	1.1 ± 0	0.9 ± 0	1.5 ± 0.1	1.5 ± 0.1	1.5 ± 0.1
DMA 18:1	0.2 ± 0.1	0.3 ± 0.1	0.4 ± 0	0.1 ± 0.1	0.6 ± 0	0.5 ± 0.1
18:0	17.3 ± 0.2	17.5 ± 0.3	18.5 ± 0.3	19.4 ± 0.6	17.9 ± 0.3	20.5 ± 0.8
18:1	11.1 ± 0.2	12.8 ± 0.2	9.9 ± 0.1	10.3 ± 0.3	12.1 ± 0.3	10.1 ± 0.2
18:2ω6	14.1 ± 0.3	13.9 ± 0.3	15.8 ± 0.4	7.2 ± 0.2	7.5 ± 0.3	8.5 ± 0.2
18:3ω6	–	–	–	0.3 ± 0.2	0.1 ± 0	0.3 ± 0.2
18:3ω3	–	–	–	–	–	–
20:0	0.2 ± 0	0.2 ± 0	0.2 ± 0	0.2 ± 0	0.2 ± 0	0.2 ± 0
20:1	0.6 ± 0.2	0.4 ± 0.1	0.3 ± 0	0.5 ± 0.2	0.3 ± 0	0.5 ± 0.1
20:2ω6+20:3ω9	0.2 ± 0	0.2 ± 0	0.5 ± 0	0.3 ± 0	0.2 ± 0	0.3 ± 0
20:3ω6	0.8 ± 0.1	0.7 ± 0.1	0.5 ± 0	0.9 ± 0.1	0.6 ± 0	0.6 ± 0
20:4ω6	31.7 ± 0.4	30.5 ± 0.2	34.3 ± 0.4	30.5 ± 0.6	30.2 ± 0.4	29.7 ± 1.6
22:0	–	–	–	–	–	–
22:1+20:5ω3	1.5 ± 0.2	1.3 ± 0.1	0.1 ± 0.1	0.6 ± 0.2	0.9 ± 0.1	0.1 ± 0
22:4ω6	0.2 ± 0	0.2 ± 0	0.4 ± 0	0.5 ± 0.1	0.4 ± 0	0.8 ± 0.1
24:0	0.6 ± 0.1	0.6 ± 0.1	0.9 ± 0.1	2.0 ± 0.6	1.4 ± 1.0	1.7 ± 0.1
24:1+22:5ω6	0.1 ± 0	0.1 ± 0	0.1 ± 0	0.4 ± 0.1	0.4 ± 0	0.5 ± 0.1
22:5ω3	0.1 ± 0	0.2 ± 0	0.1 ± 0	0.2 ± 0	0.3 ± 0	0.1 ± 0
22:6ω3	2.5 ± 0.1	2.4 ± 0.1	2.1 ± 0.1	2.4 ± 0.2	2.9 ± 0.2	2.2 ± 0.2

Data are as described in Table 2.

TABLE 6

Major Fatty Acids of the Phospholipids of the Mitochondrial and Microsomal Membrane Fractions from the Brain of Rats Fed Lipid-Supplemented Diets for 20 Weeks

Fatty acid (%; w/w)	Mitochondria			Microsomes		
	REF.	SAT.	UNSAT.	REF.	SAT.	UNSAT.
14:0	0.1 ± 0	0.1 ± 0	0.1 ± 0	0.1 ± 0	0.2 ± 0	0.1 ± 0
DMA 16:0	2.3 ± 0.2	2.4 ± 0.1	2.5 ± 0.1	2.3 ± 0.1	2.5 ± 0.2	2.4 ± 0.1
16:0	17.0 ± 0.6	16.9 ± 0.8	15.8 ± 0.1	26.2 ± 0.3	26.0 ± 1.5	26.8 ± 1.9
16:1	1.9 ± 0.4	2.3 ± 0.1	2.0 ± 0.1	–	–	–
17:0	0.4 ± 0	0.5 ± 0	0.4 ± 0	0.5 ± 0	0.5 ± 0	0.4 ± 0.1
DMA 18:0	3.0 ± 0.2	2.9 ± 0.1	3.2 ± 0.2	3.6 ± 0.2	3.0 ± 0.2	3.2 ± 0.3
DMA 18:1	–	–	–	–	–	–
18:0	19.3 ± 0.2	18.9 ± 0.3	19.9 ± 0.4	17.8 ± 0.1	17.3 ± 0.5	17.7 ± 0.5
18:1	25.1 ± 0.2	24.7 ± 0.2	23.8 ± 0.2	18.1 ± 0.1	18.3 ± 0.2	17.7 ± 0.4
18:2ω6	2.4 ± 0.3	2.1 ± 0.1	2.4 ± 0.1	1.5 ± 0.2	1.5 ± 0.1	1.9 ± 0.1
18:3ω6	0.2 ± 0.2	0.7 ± 0.2	0.4 ± 0.2	0.3 ± 0.1	0.6 ± 0.2	0.1 ± 0.1
18:3ω3	–	–	–	–	–	–
20:0	0.3 ± 0.1	0.3 ± 0	0.3 ± 0	0.2 ± 0	0.2 ± 0	0.2 ± 0
20:1	2.3 ± 0.1	2.3 ± 0.1	2.3 ± 0.1	1.3 ± 0.1	1.4 ± 0.2	1.1 ± 0.1
20:2ω6+20:3ω9	0.2 ± 0	0.3 ± 0	0.3 ± 0	0.2 ± 0	0.2 ± 0	0.2 ± 0
20:3ω6	0.3 ± 0	0.3 ± 0	0.3 ± 0	0.2 ± 0	0.3 ± 0.1	0.2 ± 0.1
20:4ω6	8.4 ± 0.3	8.3 ± 0.2	9.1 ± 0.2	8.1 ± 0	8.0 ± 0.1	8.5 ± 0.2
22:0	–	–	–	–	–	–
22:1+20:5ω3	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0	0.1 ± 0.1	0.3 ± 0.2	0.2 ± 0.2
22:4ω6	2.6 ± 0.2	2.5 ± 0.1	2.5 ± 0.1	2.7 ± 0.1	2.6 ± 0.1	2.6 ± 0.1
24:0	0.5 ± 0.2	0.9 ± 0.1	1.1 ± 0.1	0.4 ± 0.1	0.5 ± 0.2	1.1 ± 0.1
24:1+22:5ω6	0.9 ± 0.1	1.0 ± 0.2	0.9 ± 0.1	0.1 ± 0	0.2 ± 0	0.2 ± 0
22:5ω3	–	–	–	0.1 ± 0	0.1 ± 0	0.1 ± 0
22:6ω3	12.1 ± 0.3	12.0 ± 0.3	11.9 ± 0.2	16.1 ± 0.3	16.3 ± 0.6	15.2 ± 0.9

Data are as described in Table 2.

relation to changes in the total saturated, unsaturated, ω6 and ω3 unsaturated fatty acids and the unsaturation index is shown in Table 7.

In general, the dietary lipid treatments had little effect on the ratio of saturated to unsaturated fatty acids in the lipids of the various samples examined, despite large differences in this ratio in the diet. For the plasma lipids, the increase in total unsaturated fatty acids upon feeding the more unsaturated diet was due primarily to an increase in the proportion of 18:2ω6. Although small changes in the value of the unsaturation index in both the SAT. and UNSAT. fed animals were seen in some membranes of the heart and liver, generally little change in this parameter was evident. The various dietary lipid treatments had little effect on the overall level of unsaturated fatty acids, but they did have a significant effect on the type of unsaturated fatty acids present in the membrane lipids, particularly with regard to the proportions of the ω6 and ω3 series of unsaturated fatty acids. In general, the changes observed in the ω6 and ω3 unsaturated fatty acids were in opposite directions, the UNSAT. diet elevating the proportion of the ω6 unsaturated fatty acids and the SAT. diet elevating the proportion of the ω3 unsaturated fatty acids.

With regard to the changes in the proportion of the ω6 unsaturated fatty acids as a result of the 2 dietary lipid treatments, it was apparent that the greatest change occurred in the plasma lipids (25% net change). Relatively smaller changes were evident in the various subcellular membrane lipid samples, with the extent of change for the heart, liver and erythrocyte membrane ω6 unsaturated fatty acids (2 to 12% net change) being greater than that observed for the kidney and the brain (Table 7).

Certain tissue-specific responses also were observed in the type of 6 unsaturated fatty acids undergoing the greatest change. Thus, in response to increased dietary 18:2, the levels of linoleic acid increased in plasma and red blood cells without any change in 20:4 levels. In contrast, the SAT. diet induced reductions in plasma 18:2 and 20:4 levels. The mitochondrial and microsomal membranes of heart and liver shifted in a similar way in response to diet, in that both SAT. and UNSAT. diets resulted in decreased levels of 18:2 and increased levels of 20:4. The UNSAT. diet evoked the greatest response. Although kidney membranes showed little response to the SAT. diet, small increases in both 18:2 and 20:4 were observed upon feeding the UNSAT. diet.

The predominant fatty acid of the ω3 series to undergo change as a result of the 2 dietary

TABLE 7

Fatty Acid Composition of the Plasma Lipids and the Phospholipids Isolated from Various Membrane Fractions from Rats Fed Lipid-Supplemented Diets for 20 Weeks

Parameter	Diet					
	REF.		SAT.		UNSAT.	
	Blood lipids					
	Plasma	RBC	Plasma	RBC	Plasma	RBC
Total sat. FA	32.5	50.9	32.8	49.0	29.9	50.5
Total unsat. FA	67.5	49.1	67.2	51.0	70.1	49.5
Total ω6 unsat. FA	41.1	31.7	30.3	31.0	51.8	35.5
Total ω3 unsat. FA	4.6	5.1	4.6	5.2	1.8	3.1
Total DMA	0.5	7.6	0.5	6.8	0.2	6.1
ω6/ω3	8.9	6.2	6.6	6.0	29.0	11.4
Unsat. index	171	151	149	149	174	149
	Liver membranes					
	Mito.	Micro.	Mito.	Micro.	Mito.	Micro.
Total sat. FA	35.6	40.0	36.3	37.9	36.0	40.7
Total unsat. FA	64.4	60.0	63.7	62.1	64.0	59.3
Total ω6 unsat. FA	41.4	38.6	39.8	37.8	46.2	43.9
Total ω3 unsat. FA	8.7	7.2	9.5	8.7	7.6	6.2
Total DMA	0.6	0.7	0.4	0.6	0.4	0.4
ω6/ω3	4.7	5.4	4.2	4.3	6.0	7.0
Unsat. index	200	186	206	197	214	202
	Heart membranes					
	Mito.	Micro.	Mito.	Micro.	Mito.	Micro.
Total sat. FA	34.0	36.7	34.0	35.8	37.2	37.4
Total unsat. FA	66.0	63.3	66.0	64.2	62.8	62.6
Total ω6 unsat. FA	38.5	35.3	34.4	34.0	41.3	39.6
Total ω3 unsat. FA	16.3	13.8	18.7	17.0	12.2	10.8
Total DMA	3.4	4.6	4.4	5.2	4.0	4.3
ω6/ω3	2.4	2.6	1.8	2.0	3.4	3.7
Unsat. index	218	203	232	222	213	203
	Kidney membranes					
	Mito.	Micro.	Mito.	Micro.	Mito.	Micro.
Total sat. FA	35.0	45.8	35.1	43.7	35.6	46.0
Total unsat. FA	65.0	54.2	64.9	56.3	64.4	54.0
Total ω6 unsat. FA	46.9	39.4	45.3	38.8	51.0	39.9
Total ω3 unsat. FA	2.6	2.6	2.6	3.1	2.1	2.3
Total DMA	2.9	4.4	2.7	4.1	2.6	4.4
ω6/ω3	18.1	15.1	17.5	12.4	23.8	17.2
Unsat. index	190	170	185	173	197	168
	Brain membranes					
	Mito.	Micro.	Mito.	Micro.	Mito.	Micro.
Total sat. FA	42.9	51.2	42.9	50.3	43.3	52.0
Total unsat. FA	57.1	48.8	57.1	49.7	56.7	48.0
Total ω6 unsat. FA	14.0	12.9	13.9	12.9	14.8	13.3
Total ω3 unsat. FA	12.1	16.2	12.0	16.3	11.9	15.2
Total DMA	5.3	5.9	5.3	5.6	5.7	5.6
ω6/ω3	1.2	0.8	1.2	0.8	1.2	0.9
Unsat. index	155	165	155	167	157	160

Data are as described in Table 2. The unsaturation index is $\Sigma[(a)(b)]$ where a is the relative percentage of each unsaturated fatty acid and b is the number of double bonds for that particular fatty acid. DMA, dimethyl acetal derivative.

lipid treatments was docosahexaenoic acid (22:6ω3) (Tables 2 to 6). In comparison to the UNSAT. dietary animals, the SAT. diet increased the proportion of the ω3 series unsaturated fatty acids, with the greatest difference being observed in the phospholipids of the 2 membrane fractions from liver, heart and kidney. Differences in the proportion of the ω3 unsaturated fatty acids in brain membrane phospholipids were relatively small.

The dietary-induced changes in the type of unsaturated fatty acids present in the membrane phospholipids are best viewed in terms of changes in the ω6/ω3 ratio of unsaturated fatty acids (Table 7). In all instances this ratio was elevated on feeding the UNSAT. diet and decreased on feeding the SAT. diet relative to the value obtained on feeding the REF. diet. The extent of change in the ω6/ω3 unsaturated fatty acid ratio differed between the various tissues examined. The greatest response was observed in the fatty acids of the plasma lipids, where a 4-fold change in the value of this ratio between SAT. and UNSAT. dietary treated rats was evident. In contrast, the phospholipid fatty acids of the mitochondrial and microsomal membranes from all organs exhibited a one- to 2-fold change in the ω6/ω3 unsaturated fatty acid ratio, with the exception of the lipids from the membrane fractions from brain tissue which were virtually unchanged (Table 7). In comparison to the REF. diet, the lipid-supplemented diets were equally effective in altering the ω6/ω3 unsaturated fatty acid ratio away from the value exhibited by the REF. group for each of the respective samples examined, except those of the erythrocyte membrane. For erythrocyte membrane phospholipid fatty acids, the ω6/ω3 ratio was altered only in response to the UNSAT. diet.

DISCUSSION

This study was designed to examine the effects of dietary lipids on the fatty acid composition of microsomal and mitochondrial membrane phospholipids. In most of the tissues examined the same general findings were observed, and these can be summarized as follows. First, the proportion of saturated fatty acids in the membrane phospholipids was affected only marginally by dietary lipid manipulation. Where small changes did occur, it was apparent that increased dietary lipid saturation often caused small decreases in the proportion of saturated fatty acids, while increases in dietary polyunsaturates induced an opposite effect. Second, increasing the ratio of one class of dietary unsaturated fatty acid increased the ratio of that class of unsaturated fatty acids in the membrane lipids. For example, the diet with the highest ω6/ω3 unsaturated ratio, i.e. the UNSAT. diet, induced a change in the membrane unsaturated fatty acids toward an increased ω6/ω3 ratio. The diet characterized by the lower ω6/ω3 ratio, the SAT. diet, had the opposite effect. Third, the value for the unsaturation index of the membrane phospholipids was independent of the nature of the dietary lipid intake.

An increase in the dietary 18:2 intake increased the proportion of 6 unsaturated fatty acids in the membrane phospholipids, but did not lead to an increase in the total proportion of unsaturated fatty acids in the various membranes. In general terms, although the level of ω6 unsaturated fatty acids increased in certain membrane lipids, this was balanced by decreases in the proportion of both monounsaturated fatty acids and the ω3 series of unsaturated fatty acids, particularly docosahexaenoic acid (22:6). The effect of increasing dietary 18:2ω6 on total ω6 fatty acids was predictable with regard to the results obtained by other workers (2,4,15). The increase in ω6 fatty acids in most tissues was due mainly to increases in 20:4ω6, with the exception of samples from plasma and brain. For the brain, which is noted for its lack of response to dietary manipulation (5,15), only minimal changes in the proportion of 18:2ω6, 20:4ω6 and 22:4ω6 were observed. Of the other tissues examined, the liver elicited the greatest response, followed by the lipids from the various membranes from the heart and kidney. These results confirm the results of other workers (2,7,8,10,13-15) who have demonstrated that the composition of the membrane lipids is modulated by dietary long chain fatty acids even when the diet is adequate in all nutrients.

It is well established that several distinct pathways exist for the metabolic conversion of unsaturated fatty acids, these being denoted as the ω9 (oleate), ω6 (linoleate) and ω3 (linolenate) pathways. Important features of these pathways are, first, they are noninterconvertible and the fatty acids of one series cannot give rise to fatty acids of another series. Second, the competition for further unsaturation of 18 carbon unsaturated fatty acids is known to be in the order of ω3 > ω6 > ω9 (28). Third, to a large extent the factors controlling the level of polyunsaturated fatty acids in tissue lipids are a combination of substrate affinity and availability. The effects of substrate concentrations on the relative rates of synthesis by these 3 pathways of polyunsaturated fatty acid synthesis have been investigated both in vivo and in

vitro (29-33).

In neither of the 2 dietary manipulated groups was the level of ω3 fatty acids intentionally varied, and both lipid-supplemented diets contained a level of 18:3ω3 at about 1% (w/w) of the total fatty acid content. However, by increasing the level of the saturated fatty acid in the SAT. diet at the expense of ω6 polyunsaturated fatty acids, the ω6/ω3 ratio of the SAT. diet was lowered to 5 from about 50 in the UNSAT. diet. This reinforced the fact that in addition to the change in the proportion of saturated fatty acids, the change in the dietary lipids was a change primarily in the ω6/ω3 ratio rather than in the individual proportions of unsaturated fatty acids from each class. The effect of the SAT. diet on all tissues was to increase the proportion of ω3 unsaturated fatty acids and lower the proportion of ω6 unsaturated fatty acids. In all cases, any increase in the level of ω3 polyunsaturates due to this dietary treatment was the result of increases in the level of 22:6ω3 which presumably was synthesized from dietary 18:3ω3.

Where the supply of dietary ω3 fatty acids is nonlimiting, the role of 22:6ω3 in modulating the unsaturation index requires special comment. The compensatory variation in the levels of docosahexaenoic acid, in response to oscillating levels of ω6 fatty acids in particular, has been reported by other workers (10,34) and is confirmed by our own results. Although monounsaturates are involved in the maintenance of the unsaturation index, their contribution is relatively small. By far the greatest contributors to the unsaturation index are the ω6 and ω3 fatty acids. Since 22:6 constitutes over 90% of the total ω3 acids present in most membranes, the key regulatory role of docosahexaenoate is apparent. Obviously where dietary ω6 and ω3 fatty acids are unavailable, such as in fat-free diets, this compensatory role is taken over by monounsaturated fatty acids and the ω9 unsaturated fatty acid, 20:3.

The mechanism by which the levels of the ω6 fatty acids, principally 20:4, and the ω3 fatty acids, principally 22:6, act to maintain the unsaturation index is unclear; the process does not appear to involve a dilution of one or more fatty acids by the other. Although in vitro studies have established the effectiveness of 22:6ω3 as an inhibitor of the various desaturase enzymes (35), there is little direct evidence to support this concept from in vivo studies. The early work of Mohrhauer and Holman (29) clearly established the competitive nature of the inhibition of each of the 3 major pathways by the substrate of each fatty acid family. They concluded that the composition of the tissue polyunsaturated fatty acids is dependent upon the dietary supplies of their precursors and is regulated by competitive inhibition(s) of their metabolism. Whether any other feedback mechanism exists to allow for fine tuning of the membrane fatty acid profile and thus maintenance of the overall fluidity of the membrane is unknown. However, recent results obtained with cultured hamster kidney cells (31) indicate that while the presence of 18:2ω6 in the culture medium resulted in replacement of monoenes by 18:2ω6 at position 1 of membrane phosphatidylcholine, the presence of 20:3ω6 or 20:4ω6 resulted in these same monoenes being replaced by saturates. These results may help to explain the paradox whereby diets which are rich in 18:2ω6 and cause increased levels of 20:3ω6 and 20:4ω6 result in slightly higher levels of saturates in some membranes.

We believe our data illustrate an important homeostatic principle in which the level of membrane lipid saturation/unsaturation is buffered against transient changes which may be induced by alteration in the nature of the dietary lipid intake. Many reports (1-10,27-29, 34) confirm such a concept of lipid homeostasis. One recent study confirms our findings in the microsomes and mitochondrial membranes of the rat, although in that study the phospholipid fraction was not examined (15). Furthermore, even the work on rats raised on fat-free diets (34,36) confirms that there is a maintenance of both the level of lipid saturation and the unsaturation index to within a few per cent, despite the unavailability of both ω6 and ω3 unsaturated fatty acids in the diet. Although we did not attempt to determine the dynamic nature of these changes in membrane lipid composition, we have established in separate experiments that the changes that occur after 20 weeks do not change significantly after 12 mo of dietary lipid treatment (data not shown). It has been shown by others (37) that changes can occur in the fatty acid component of certain phospholipids of rat liver membranes within hours of dietary lipid changes.

In summary, our data clearly demonstrate that dietary fats can change the composition of membrane phospholipids in only a very restricted sense. The proportion of saturated fatty acids in membrane phospholipids appears to remain relatively constant, regardless of the dietary treatment, and may be dictated by the specificity of the *sn*-1 position of the phospholipid molecule for saturated fatty acids. Our data also suggest that there is competition for the *sn*-2 position of the phospholipid molecule

by long chain ω6 and ω3 polyunsaturated fatty acids, particularly 20:4ω6 and 22:6ω3. Whether such a homeostatic control of the unsaturation index of the membrane lipids allows for the maintenance of more than just the physical properties of the bulk lipid phase of the membrane remains to be determined. The question as to the consequences of this lipid homeostasis in terms of possible modulation of various membrane-associated enzyme activities, is also an important aspect for consideration with regard to the potential effects of dietary lipids on various physiological processes.

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REFERENCES

1. Holman, R.T. (1964) Fed. Proc. 23, 1062-1067.
2. De Schrijver, R., and Privett, O. (1982) J. Nutr. 112, 619-626.
3. FAO Food and Nutrition Paper No. 3 (1978).
4. Kramer, J.K.G. (1980) Lipids 15, 651-660.
5. Purvis, J.M., Clandinin, M.T., and Hacker, R.R. (1982) Comp. Biochem. Physiol. 72B, 195-199.
6. Caster, W.O., Andrews, J.W., Mohrhauer, H., and Holman, R.T. (1976) J. Nutr. 103, 1809-1816.
7. Farquhar, J.W., and Ahrens, E.H. (1963) J. Clin. Invest. 42, 675-685.
8. Mohrhauer, H., and Holman, R.T. (1963) J. Nutr. 81, 67-74.
9. Innis, S.M., and Clandinin, M.T. (1980) J. Nutr. 110, 1006-1013.
10. Paturneau-Jonas, M., Durand, G., Nouvelot, A., Masson, M., and Bouvre, J.M. (1982) Reprod. Nutr. Develop. 22, 193-200.
11. Morand, O., Chanez, C., Masson, M., Dumont, O., Flexor, M.A., Baumann, N., and Bouvre, J.M. (1982) Ann. Nutr. Metab. 26, 111-120.
12. Kurata, N., and Privett, O.S. (1980) Lipids 15, 1029-1036.
13. Kurata, N., and Privett, O.S. (1980) Lipids 15, 512-518.
14. Innis, S.M., and Clandinin, M.T. (1981) Biochem. J. 193, 155-167.
15. Tahin, Q.S., Blum, M., and Carafoli, E. (1981) Eur. J. Biochem. 121, 5-13.
16. Clandinin, M.T. (1978) J. Nutr. 108, 273-281.
17. Chapman, D. (1975) in Cell Membranes, Biochemistry, Cell Biology and Pathology (Weissman, G. and Claiborne, R., eds.) pp. 13-22, Hospital Practice Publishing Co., New York.
18. Abuirmeileh, N.M., and Elson, C.E. (1980) Lipids 15, 918-924.
19. Innis, S.M., and Clandinin, M.T. (1981) Biochem. J. 198, 167-175.
20. McMurchie, E.J., and Raison, J.K. (1979) Biochim. Biophys. Acta 554, 364-374.
21. McMurchie, E.J., Gibson, R.A., Abeywardena, M.Y., and Charnock, J.S., Biochim. Biophys. Acta 727, 163-169.
22. McMurchie, E.J., Abeywardena, M.Y., Charnock, J.S., and Gibson, R.A. (1983) Biochim. Biophys. Acta 760, 13-24.
23. Galli, C., Agradi, E., Petroni, A., and Tremoli, E. (1982) Lipids 16, 165-172.
24. Galli, C., Spagnuolo, C., Bosisio, E., Tosi, L., Folco, G.C., and Galli, G. (1978) in Advances in Prostaglandin and Thromboxane Research (Coceani, F. and Olley, P.M., eds.) Vol. 4, pp. 181-189, Raven Press, New York.
25. Olley, P.M., and Coceani, F. (1980) Am. J. Dis. Child. 134, 688-696.
26. Broekhuyse, R.M. (1974) Clin. Chim. Acta 51, 341-343.
27. Pudelkewicz, C., Seufert, J., and Holman, R.T. (1968) J. Nutr. 94, 138-146.
28. Brenner, R.R., and Peluffo, R.O. (1966) J. Biol. Chem. 241, 5213-5219.
29. Mohrhauer, H., and Holman, R.T. (1963) J. Nutr. 81, 67-74.
30. Mohrhauer, H., and Holman, R.T. (1963) J. Lipid Res. 4, 151-159.
31. Stern, W., and Pullman, M.E. (1983) J. Biol. Chem. 258, 11128-11135.
32. Brenner, R.R. (1974) Molec. Cell. Biochem. 3, 41-52.
33. Brenner, R.R., and Peluffo, R.O. (1969) Biochim. Biophys. Acta 176, 471-479.
34. Iritani, N., and Fujikawa, S. (1982) J. Nutr. Sci. Vitaminol. 28, 621-629.
35. Sprecher, H. (1977) in Polyunsaturated Fatty Acids (Kunau, W.H. and Holman, R.T., eds.) pp. 1-18, Am. Oil Chem. Soc., Champaign, IL.
36. Odutuga, A.A. (1981) Int. J. Biochem. 13, 1035-1038.
37. Flick, P.K. (1981) Nutr. Rep. Int. 24, 667-674.

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Photosensitized Oxidation of Methyl Linolenate Monohydroperoxides: Hydroperoxy Cyclic Peroxides, Dihydroperoxides and Hydroperoxy bis-Cyclic Peroxides[1]

W.E. NEFF and **E.N. FRANKEL,*** *Northern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Illinois 61604*

ABSTRACT

To elucidate the biological and flavor significance of lipid secondary oxidation products, cyclic peroxides were prepared by photosensitized oxidation of the hydroperoxides in autoxidized methyl linolenate. The oxidation product was fractionated by silicic acid chromatography, followed by high pressure liquid chromatography (HPLC) on a microporous silica column. Products characterized by thin layer chromatography (TLC), gas chromatography (GC), ultraviolet (UV), infrared (IR), nuclear magnetic resonance (NMR) and mass spectroscopy (MS) included 6-membered cyclic peroxides (9-hydroperoxy-10,13-epidioxy-11, 15- and 16-hydroperoxy-12,15-epidioxy-9, 13-octadecadienoates), 15-membered cyclic peroxides (9-hydroperoxy-10,12-epidioxy-13,15- and 16-hydroperoxy-13, 5-epidioxy-9,11-octadecadienoates), dihydroperoxides (9,12-; 9,16-; 10,12-; 10,15-; 10,16-; 13,15-; 13,16-dihydroperoxy octadecatrienoates) and hydroperoxy bis-cyclic peroxides, each with one 5- and one 6-membered ring (9-hydroperoxy-10,12,13,16-bis-epidioxy-14- and 16-hydroperoxy-9,12,13, 15-bis-epidioxy-10-octadecenoates). The 6-membered cyclic peroxides are formed by 1,4-addition of singlet oxygen to the conjugated diene system in 9- and 16-linolenate hydroperoxide isomers after their isomerization to the *trans,trans* configuration. The bis-cyclic peroxides are formed by 1,4-addition of singlet oxygen to the hydroperoxy 5-membered cyclic peroxides derived from the 12- and 13-linolenate hydroperoxide isomers. Secondary oxidation products similar to those identified in this study previously have been shown to be important precursors of volatile compounds that may contribute to flavor deterioration of fat-containing foods.

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INTRODUCTION

In previous studies, secondary products characterized from autoxidized and singlet oxygenated methyl linoleate and linolenate included 5-membered hydroperoxy epidioxides (1-8) dihydroperoxides (5,7) and hydroperoxy bicycloendoperoxides (4,7). Recently, we identified 6-membered hydroperoxy epidioxides in the products obtained from linoleate monohydroperoxides (9-OOH and 13-OOH) subjected to photosensitized oxidation (9). These secondary oxidation products were shown to be important precursors of volatile compounds associated with odor and flavor deterioration of fats (5,7,9-13). The suggestion that 6-membered hydroperoxy epidioxides are likely precursors of pentyl furan (13) was confirmed recently in our studies of thermal decomposition of these cyclic peroxides (9). Hydroperoxy epidioxides also have been shown to be active in the formation of fluorescence with DNA (14), and thus may have biological activity due to their crosslinking properties.

This paper extends our previous studies (9) to the photosensitized oxidation of the conjugated hydroperoxides from autoxidized methyl linolenate (9-, 12-, 13-, and 16-OOH). New compounds identified in this work include 6-membered monocyclic and bis-cyclic peroxides with both 5-membered and 6-membered rings in the same molecule.

*To whom correspondence should be addressed.

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EXPERIMENTAL

Materials and Methods

Previous methods were used to prepare pure methyl linolenate (15) and monohydroperoxides from autoxidized linolenate (3). The mixture of 9-, 12-, 13- and 16-monohydroperoxide isomers was subjected to oxidation by singlet oxygen ($^1O_2$) under the same conditions of photosensitized oxidation as before (5,9). After photosensitized oxidation at 0 C for 10 hr, TLC indicated that about 50% of the monohydroperoxides were converted into secondary oxidation products.

Separation and Characterization

The reaction mixture was separated by silicic acid chromatography as described previously (3). Selected fractions, containing hydroperoxy epidioxides, dihydroperoxides or hydroperoxy

bis-epidioxides, were resolved by HPLC as detailed previously (7,9). The oxidation products were characterized by GC, TLC, UV, IR, NMR, gas chomatography-mass spectrometry (GC-MS) and MS (7,9). The isomeric composition of linolenate monohydroperoxides was determined by HPLC of the hydroxy derivatives obtained after $NaBH_4$ reduction (16).

RESULTS AND DISCUSSION

The monohydroperoxide mixture separated from autoxidized methyl linolenate was photooxidized with methylene blue. After a 10-hr reaction with $^1O_2$ in CH_2Cl_2 at 0 C, the secondary oxidation products were separated by silicic acid chromatography and identified for functionality by comparing their TLC properties with products previously characterized from photosensitized oxidation of methyl linolenate (7). Quantitative analysis of the products obtained by silicic acid chromatography showed the following relative weight percentages: nonpolar material, presumably keto trienes (7), 4.5%; unreacted monohydroperoxides, 46.2%; hydroperoxy 5- and 6-membered epidioxides, 9.7%; dihydroperoxides, 19.2%; hydroperoxy bis-epidioxides, 5.2% and unidentified polar substances, 15.2%. The mono- and dihydroperoxides and hydroperoxy mono- and bis-epidioxides were identified further after purification by HPLC.

6- and 5-Membered Hydroperoxy Epidioxides

The mixture of hydroperoxy monoepidioxides separated by silicic acid chromatography was resolved by HPLC (Fig. 1) into the following components: unreacted monohydroperoxides, 30%; methyl 9-hydroperoxy-10,13-epidioxy-11, 15- and 16-hydroperoxy-12,15-epidioxy-9,13-octadecadienoates (I, II), 12%; 9-hydroperoxy-10,12-epidioxy-13,15- (III), 17%; 16-hydroperoxy-13,15-epidioxy-9,11-octadecadienoates (IV), 26% and unidentified material, 15%. The structures established chromatographically and spectrally for these compounds are given in Figure 2.

TLC (silica, diethyl ether/hexane, 60:40) of HPLC fractions I + II, UV inactive, R 0.79; fraction III, UV active, R 0.72; and fraction IV, UV active, R 0.69 relative to methyl linolenate monohydroperoxides. Fractions I-IV were all peroxide positive.

IR (CS_2) of fraction I + II: 3635-3300 (bonded C-OOH), 3005 (olefinic-H), 965 (isolated *trans* double bond); fraction III: 3600-3350 (bonded C-OOH), 3010 (olefinic-H), 995 (S), 945 (W) (conjugated diene); fraction IV: 3600-3350 (bonded C-OOH), 3000 (olefinic-H),

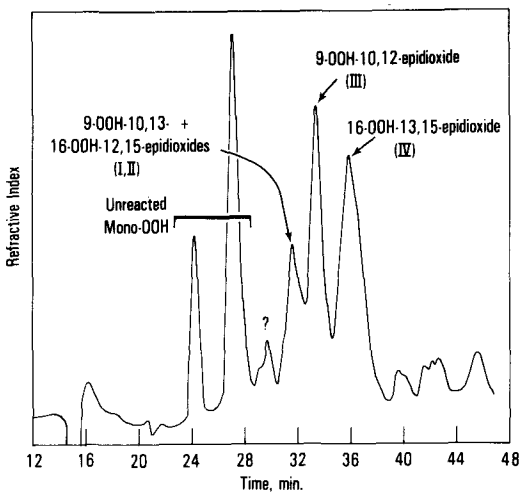


FIG. 1. 10-μm silica HPLC chromatogram of hydroperoxy epidioxides from singlet oxygenated methyl linolenate monohydroperoxides [flow 4 ml/min; mobile phase, hexane/CH_2Cl_2/ethyl acetate (7:4:1, v/v/v); refractive index detector X 8, column temperature 20 C].

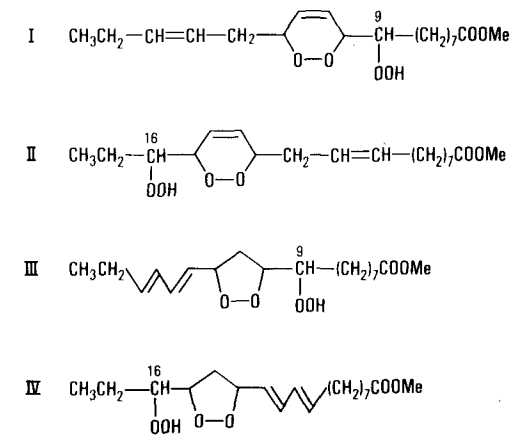


FIG. 2. Structures of hydroperoxy epidioxides isolated by HPLC (Fig. 1).

985 (S), 948 (W) (conjugated diene) cm^{-1}.

^{1}H-NMR (90 MHz, $CDCl_3$) of HPLC fractions I + II: 8.65 (s, 1, OOH), 5.98 [br s, 2, olefinic protons of 6-membered ring with endocyclic double bond (9)], 5.45 (m, 2, $CH_2\underline{CH}$=$\underline{CH}CH_2$), 4.65 [m, 1, proton of 6-membered ring methine carbon adjacent to exocyclic hydroperoxy bearing carbon (9)], 4.48 [m, 1, proton of 6-membered methine carbon adjacent to exocyclic methylene group (9)], 4.22 (m,1, $\underline{CH}OOH$), 3.65 (s, 3, $CO_2\underline{CH_3}$), 2.32 (t, 2, $\underline{CH_2}CO$), 2.05 (m, 2, $\underline{CH_2}C$=C), 1.6 (m, 2, $\underline{CH_2}CHOOH$), 2.0 (m, 2, $\underline{CH_2}$-C-OO-C-), 1.35 [s, 10, $(CH_2)_X$], 0.95 (t, 3, $\underline{CH_3}C$) ppm.

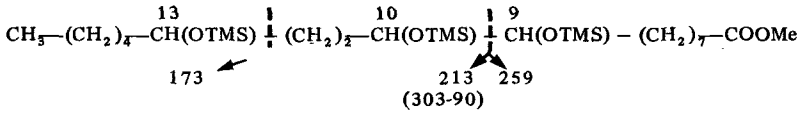
HPLC fractions III and IV showed the same ^{1}H-NMR absorptions as reported previously for 9-OOH-10,12-epidioxy-13,15- and 16-OOH-13, 15-epidioxy-9,11-octadecadienoates, respectively (3,9).

For compounds I + II, IR and ^{1}H-NMR data support the general structures of a 6-membered hydroperoxy cyclic peroxide with an endocyclic double bond, a homoallylic exocyclic double bond and an exocyclic hydroperoxide group. For III and IV, the IR and ^{1}H-NMR data support a 5-membered hydroperoxy cyclic peroxide with an exocyclic hydroperoxide group and conjugated diene system in the *trans,trans* configuration (3,8) (Fig. 2).

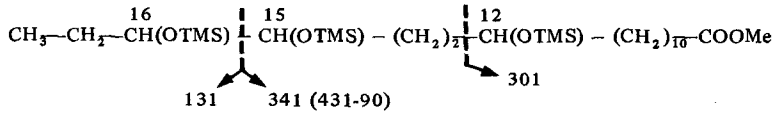
These structures were supported further by GC and GC-MS analyses of the silylated polyhydroxy derivatives. On catalytic hydrogenation followed by silylation, I gave 9,10,13- and II gave 12,15,16-tri(OTMS)stearates, identified by GC retentions relative to methyl stearate and by GC-MS (17), m/z (rel. intensity) I + II: M-(90+15), 457 (4); 73 (100); I: 259 (23), 213 (32); 172 (22) (Structure A) and for II: 341 (17), 301 (22) and 131 (38) (Structure B). On hydrogenation followed by silylation, III gave 9,10,12- and IV gave 13,15,16-tri(OTMS) stearates, identified by GC retentions relative to methyl stearate and by GC-MS (3).

Structures of I and II were further confirmed by MS of unsaturated hydroxy derivatives obtained after reduction with Ph_3P followed by silylation, m/z (rel. intensity) I + II (Fig. 2) M, 412 (1); M-16, 396 (1); m-32, 380 (2); M-90, 322 (3); 73 (72); 75 (100); I: 259 (19); 153 (15); 135 (25); 69 (22); 55 (44) (Structure C) and II 131 (22); 267 (5); 281 (1); 183 (10); 197 (8) (Structure D).

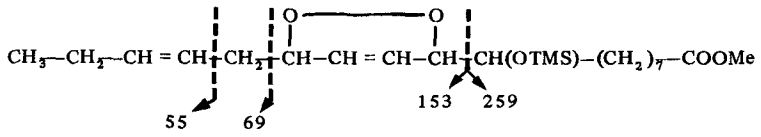
This study shows that the 6-membered hydroperoxy epidioxides may be formed by 1,4-addition of $^1O_2$ to the 1,3-diene system of the 9- and 16-monohydroperoxides of methyl linolenate (Fig. 3). According to the mechanism in Figure 3, the *trans,cis* diene hydroperoxides



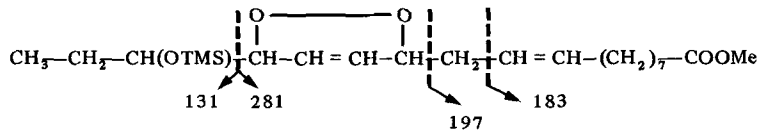
STRUCTURE A



STRUCTURE B



STRUCTURE C



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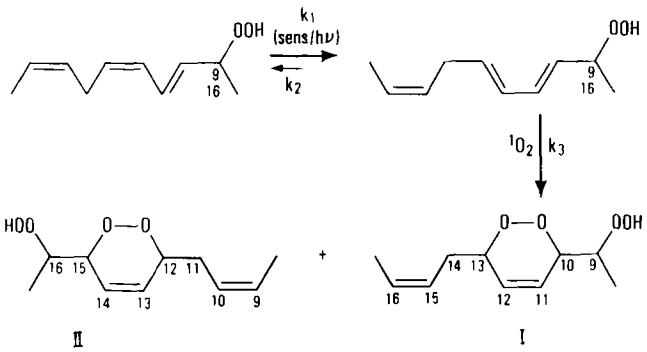


FIG. 3. Scheme for the formation of 6-membered hydroperoxy epidioxides from linolenate monohydroperoxides (sens = sensitizer).

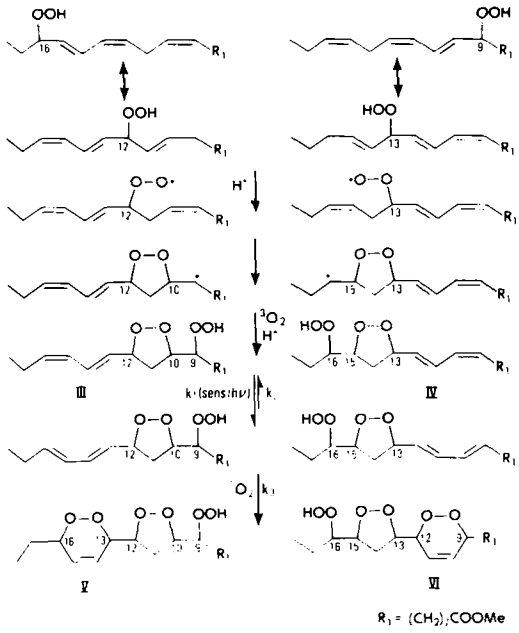


FIG. 4. Scheme for the formation of 6 5-membered hydroperoxy bis-epidioxides from linolenate monohydroperoxides (sens = sensitizer).

are isomerized to the *trans,trans* configuration before cycloaddition of $^1O_2$, as suggested previously for linoleate hydroperoxides (9). HPLC quantitative analyses of the linolenate hydroperoxides before and after reaction with $^1O_2$ indicated little change in the amount of *trans, trans* hydroperoxy diene derivatives. The ratio of *trans,trans* to *cis,trans* monohydroperoxide mixtures changed by only 2% after $^1O_2$ oxidation. These results are explained, as before (9), by a mechanism that assumes photosensitized isomerization (k_1/k_2) from *cis,trans* to *trans, trans* to be faster than 1,4-cycloaddition (k_3) of $^1O_2$ to the conjugated diene system of the linolenate hydroperoxides (Fig. 3).

Under the conditions of photosensitized oxidation, significant amounts of 5-membered hydroperoxy epidioxides also are formed, apparently by free radical cyclization of the internal 12- and 13-monohydroperoxides of methyl linolenate (Fig. 4). The higher proportion found for 5-membered cyclic peroxides (III, IV) than for 6-membered cyclic peroxides (I, II, Fig. 1) indicates that free radical cyclization of the 12- and 13-hydroperoxide isomers is more prevalent than 1,4-cycloaddition of $^1O_2$ to the 9- and 16-hydroperoxide isomers of methyl linolenate. HPLC analysis of monohydroperoxides of linolenate (16) before and after photosensitized oxidation showed only little change in the positional isomeric distribution (% before and after oxidation): 28, 30% 9-OOH; 13, 12%, 12-OOH; 18, 15% 13-OOH and 41, 43% 16-OOH. According to schemes in Figures 3 and 4, 6-member cyclic peroxides come from 9- and 16-OOH, and the 5-member cyclic peroxides come from 12- and 13-OOH. Therefore, to explain the larger concentration of 5-membered than 6-membered cyclic peroxides, one may postulate that the 9- and 16-hydroperoxides undergo isomerization to 12- and 13-hydroperoxides, as postulated by Coxon et al. (2). This positional isomerization would be significant only when pure linolenate hydroperoxides are photooxidized. During autoxidation of methyl linolenate, we previously found no evidence for this positional isomerization of hydroperoxides (3), because it would be inhibited by unreacted methyl linolenate, as reported by Porter et al. for linoleic and arachidonic acids (18,19).

Hydroperoxy bis-Epidioxides

One silicic acid chromatographic fraction, which contained a non-UV active and hydroper-

oxide positive component, had an R similar to singlet oxygenated methyl linolenate hydroperoxy bis-epidioxides (7). After HPLC purification, a fraction was obtained that was characterized by ^{1}H-NMR and by GC and GC-MS after hydrogenation and silylation as a mixture of 9- and 16-hydroperoxy bis-epidioxides with one 5-membered ring and one 6-membered ring V, VI (Fig. 4).

^{1}H-NMR (90 MHz, $CDCl_3$) of V + VI: 8.45 (s, 1, OOH), 5.68 [m, 2, olefinic protons of a 6-membered ring with endocyclic double bond (9)], 4.45 [m, 4, protons of methine carbons in the 5- and 6-member rings (7,9)], 4.08 (s, 1, CHOOH), 3.67 (s, 3, CO_2CH_3), 2.1-2.7 [m, 2, methylene protons of a 5-member ring (7)], 2.3 (t, 2, CH_2CO), 1.6 (m, 4, CH_2CHOOH/CH_2CH_3), 1.35 [m, 10, $(CH_2)_X$] and 0.95 (t,3,CH_3C) ppm.

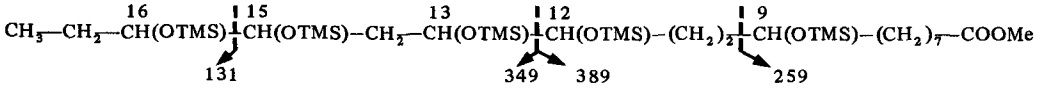
GC-MS of V and VI after hydrogenation and silylation, m/z (rel. intensity): 73 (100), 259 (18), 389 (479-90, 6%), 299 (389-90, 20%), 387 (477-90, 25%), 297 (387-90, 11%), 261 (5%), 171 (261-90, 43%), 131 (26%), 169 (259-90, 2%), 79 (169-90, 3%), 209 (299-90, 9%), 207 (297-90, 4%), and 81 (171-90, 6%) (Structures E, F).

The structures of V and VI were confirmed further by MS of hydroxy derivatives obtained after Ph_3P reduction and silylation, m/z (rel. intensity) (Fig. 4), V: 73 (100), 259 (83), 185 (22) (Structure G) and VI: 131 (94), 313 (2) (Structure H).

The hydroperoxy bis-epidioxides V and VI apparently are formed from the 12- and 13-hydroperoxides after change of configuration of the conjugated diene system from *cis,trans* to *trans,trans* (Fig. 4). This process is followed by free radical cyclization and hydroperoxidation to form first the 5-membered epidioxides, followed by 1,4-cycloaddition of $^1O_2$ to the epidioxide diene system to form the adjacent 6-membered epidioxide.

Dihydroperoxides

The silicic acid chromatographic fraction containing dihydroperoxides was resolved by HPLC into positional isomers, as described for



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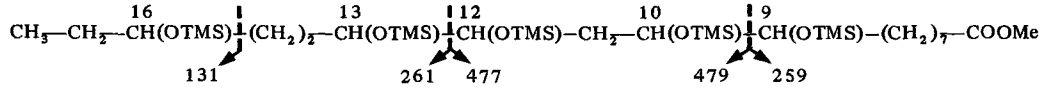

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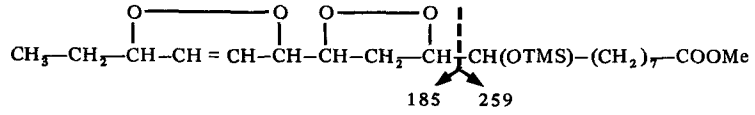
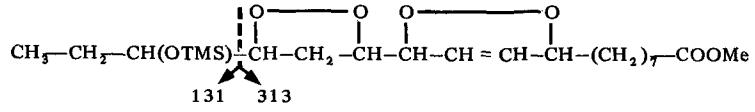


STRUCTURE G



STRUCTURE H

singlet oxygenated linolenate (7). Characterization of these isomers by HPLC retention, ^{1}H-NMR, and by GC and GC-MS of the silylated, hydrogenated derivatives showed that oxidation of linolenate monohydroperoxides with $^1O_2$ produced the same dihydroperoxides previously reported for singlet oxygenated linolenate (7). The following weight per cent composition was estimated by HPLC: 9,12 + 13,16-diOOH (4%); 10,12-diOOH (5%); 13,15-diOOH (8%); 10,16-diOOH (15%); 9,15-diOOH (15%); *trans,cis,trans*-9,16-diOOH (34%) and *trans,trans,trans* 9,16-diOOH (19%). These dihydroperoxides apparently are derived by both free radical and $^1O_2$ oxidation of linolenate monohydroperoxides (7).

We previously found that thermal decomposition of hydroperoxy cyclic peroxides and dihydroperoxides (9,10) produced volatile materials similar to those of monohydroperoxides (12) (e.g., hydrocarbons, aldehydes, furans, ketones, unsaturated ketone, aldehydes and alcohols). Therefore, the cyclic peroxides and dihydroperoxides identified in this work also would be expected to produce similar volatile products that may have flavor significance.

ACKNOWLEDGMENT

S. Hvizdos assisted with high-pressure liquid chromatography.

REFERENCES

1. Mihelich, E.D. (1980) J. Am. Chem. Soc. 102, 7141-7143.
2. Doxon, D.T., Price, K.R., and Chan, H.W.-S. (1981) Chem. Phys. Lipids 28, 365-378.
3. Neff, W.E., Frankel, E.N., and Weisleder, D. (1981) Lipids 16, 439-448.
4. O'Connor, D.E., Mihelich, E.D., and Coleman, M.C. (1981) J. Am. Chem. Soc. 103, 223-224; (1984) 106, 3577-3584.
5. Frankel, E.N., Neff, W.E., Selke, E., and Weisleder, D. (1982) Lipids 17, 11-18.
6. Frankel, E.N., Neff, W.E., and Weisleder, D. (1982) J. Chem. Soc. Chem. Commun. 599-600.
7. Neff, W.E., Frankel, E.N., and Weisleder, D. (1982) Lipids 17, 780-790.
8. Toyoda, I., Terao, J., and Matsushita, S. (1982) Lipids 17, 84-90.
9. Neff, W.E., Frankel, E.N., Selke, E., and Weisleder, D. (1983) Lipids 18, 868-876.
10. Frankel, E.N., Neff, W.E., and Selke, E. (1983) Lipids 18, 353-355.
11. Frankel, E.N., Neff, W.E., and Selke, E. (1984) Lipids 19, 790-800.
12. Frankel, E.N., Neff, W.E., and Selke, E. (1981) Lipids 16, 279-285.
13. Frankel, E.N. (1983) Prog. Lipid Res. 22, 1-33.
14. Fujimoto, K., Neff, W.E., and Frankel, E.N. (1984) Biochim. Biophys. Acta 795, 100-107.
15. Frankel, E.N., Neff, W.E., Rohwedder, W.K., Khambay, B.P.S., Garwood, R.F., and Weedon, B.C.L. (1977) Lipids 12, 1055-1061.
16. Chan, H.W.-S., and Levett, G. (1977) Lipids 12, 99-104.
17. Dommes, V., Wirtz-Peitz, F., and Kunau, W.-H. (1976) J. Chromatogr. Sci. 14, 360-366.
18. Porter, N.A., Weber, B.A., Weenen, H., and Khan, J.A. (1980) J. Am. Chem. Soc 102, 5597-5601.
19. Porter, N.A., Lehman, L.S., Weber, B.A., and Smith, K.J. (1981) J. Am. Chem. Soc. 103, 6447-6455.

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Lipid Composition of Millet (*Pennisetum americanum*) Seeds

A.U. OSAGIE[1] and **M. KATES***, *Department of Biochemistry, University of Ottawa, Ottawa, Ontario, Canada K1N 9B4*

ABSTRACT

The composition of lipids extracted from a sample of millet seeds by each of 8 solvent systems is reported. Lipid components were separated by silicic acid column and thin layer chromatography (TLC) and quantitated by analysis of fatty acid methyl esters by gas liquid chromatography (GLC), with heptadecanoic acid as internal standard. Best results were obtained by extraction with hot water-saturated butanol. Lipids extracted amounted to 7.2% of the seed dry weight and consisted of 85% neutral lipids, 12% phospholipids and 3% glycolipids. Neutral lipids contained mostly (85%) triacylglycerols and small amounts of mono- and diacylglycerols, sterols and free fatty acids. Sterols consisted of campesterol, stigmasterol and 2 unidentified sterols, occurring in the same proportions in free and esterified forms. Ten glycolipid and 10 phospholipid components were separated and characterized. Contrary to previously published observations, lysophosphatidylcholine was the major phospholipid (42%) in millet seeds; smaller amounts of phosphatidylcholine (24%), lysophosphatidylethanolamine (21%) and trace amounts of phosphatidylglycerol, phosphatidic acid, phosphatidylserine and phosphatidylinositol also were present. The major glycolipids were esterified sterol glycoside, sterol glycoside, monogalactosyldiacylglycerol, digalactosyldiacylglycerol and cerebrosides (ceramide monohexosides). *Lipids* 19:958-965, 1984.

INTRODUCTION

Millet seeds are of considerable importance in many countries as a food for human consumption. Because it is one of man's most drought tolerant food crops, millet is grown primarily in the dry sahel zone across Africa and in the semi-arid areas of India. Estimated production is about 18 million metric tons annually. With the development and testing of new varieties, improved production and processing methods and the introduction of new products, the use of this cereal may be expanded further.

The quality of millet deteriorates quickly after it has been ground into a meal, and the action of activated lipase and phospholipase on the lipid components is considered to be responsible for the deterioration (1). Odor changes were detected in millet meal stored at 19 C and 58% relative humidity for only 4-5 days (1). Carnovale and Quaglia (2) observed a 3% decrease in ether-extractable lipids on storing millet flour at 30 C and 95% relative humidity for 3 mo. Using infrared spectrometry to study the extracted lipids, they observed an increase in absorption due to the -COOH group, indicating an increase in free fatty acids and suggesting that hydrolytic as well as oxidative changes in lipids occur during storage. Commercial malters reject millet seeds because the relatively low carbohydrate and the high lipid content would contribute to poor head retention and organoleptic properties of the beer (3).

The level of petroleum ether extractable free lipids in millet cultivars varies from 3.0 to 7.4% (1,4-7). The bound lipids extractable with water-saturated butanol range from 0.58 to 0.90% (1). Prudthi and Bhatia (8) reported the composition of various polar lipid components of *Pennisetium typhoideum*, after separation by two-dimensional TLC, but some of the components were identified erroneously. Badi et al. (9) separated millet *(Pennisetium americanum* (L). K. Schum) free and bound lipids by TLC, but gave no quantitative results.

Thus, information available on the lipid components of millet is incomplete. This paper reports on the relative efficiency of different solvents in extracting lipids from millet seeds and on the composition of the individual lipids extracted.

MATERIALS AND METHODS

Millet seeds *(Pennisetum americanum)*, grown in Zaria, Northern Nigeria, were harvested in December 1982 and sun-dried within 5-7 days after harvest; they were collected on Jan. 5, 1983, packed in an air-tight plastic bag sealed in a metal tin, transported to Ottawa by air on Jan. 22, 1983 and stored in the sealed container at 4 C until analyzed. The seeds were ground in a Moulinex coffee grinder and analyzed for moisture (10); a moisture content of 7.6-7.8% was found. Portions (10g) of the ground material were immediately extracted

[1]On leave from Department of Biochemistry, University of Benin, Benin City, Nigeria.

*To whom correspondence should be addressed.

separately with each of the following solvents[2]: A) Soxhlet extraction with 250 ml n-hexane/diethyl ether (80:20, v/v) for 16 hr; B) Soxhlet extraction with 250 ml 85% aqueous methanol for 16 hr; C) Soxhlet extraction with 250 ml chloroform/methanol (2:1, v/v) for 16 hr; D) Soxhlet extraction with 250 ml ethanol/ether/water (2:2:1, v/v/v) for 16 hr; E) boiling with 250 ml ethanol/water (80:20, v/v) under reflux for 12 hr; F) boiling with 250 ml isopropyl alcohol for 30 min, then extraction 3 times with 50 ml portions of methanol/chloroform (2:1, v/v) with vigorous agitation at room temperature; G) extraction 3 times with water-saturated butanol (sample weight/solvent volume, 1:5) for 1 hr each with vigorous agitation at room temperature, and H) extraction 3 times with hot water-saturated butanol (sample weight/solvent volume, 1:5, w/v) for 1 hr each time with vigorous shaking. After each hour, the mixture was cooled to 20 C and filtered with suction.

The crude extracts were centrifuged to remove insoluble material and evaporated to dryness on a rotary evaporator. The residues were purified by extraction with chloroform/methanol/water (1:2:0.8, v/v/v) and forming a biphasic system (chloroform/methanol/water, 1:1:0.9, v/v/v) by addition of chloroform and water (11); the chloroform layer was diluted with benzene and brought to dryness on a rotary evaporator. The purified lipids were fractionated by silicic acid column chromatography into neutral lipid (NL), glycolipid (GL) and phospholipid (PL) fractions (12) and each fraction was examined by TLC on pre-coated silica gel plates (J.T. Baker, Phillipsburg, New Jersey) in the following solvent systems: I) diethyl ether/benzene/ethanol/acetic acid (40:50:2:0.2, v/v/v/v); II) n-hexane/diethyl ether (96:4, v/v); III) chloroform/methanol/H_2O (65:25:4, v/v/v); IV) chloroform/methanol/conc. ammonia (65:35:5, v/v/v); V) chloroform/methanol/acetone/diethylamine/H_2O (120:35:37.5:6:4.5, v/v/v/v/v), and VI) chloroform/acetone/methanol/acetic acid/H_2O (10:4:2:2:1, v/v/v/v/v). All solvents were freshly distilled before use.

Neutral lipids were separated by development of the plates first with solvent system I, then, after air-drying, in the same direction with system II. Polar lipids were separated by one- or two-dimensional TLC using systems III and IV. The glycolipids were resolved in solvent system V while the phospholipids were resolved in solvent system VI. Lipids were detected on TLC plates by charring with 50% H_2SO_4 or by spraying with specific spray reagents (12). Identification of polar lipid components was established by use of specific spray reagents (12) and comparison of their R_f values with those of authentic wheat lipids (13).

Fatty acid methyl esters (FAME) were prepared by methanolysis of the lipid fraction or component in 2.5% methanolic HCl (gaseous) (12) and were analyzed on a Pye-Unicam gas chromatograph equipped with dual flame ionization detectors on a glass column (2m × 4mm) packed with 10% SP2330 polyester on 80/100 mesh chromosorb W-AW support (Supelco Inc., Bellefonte, Pennsylvania) and operated at 225 C under a nitrogen flow rate of 30 ml/min. Each major lipid fraction obtained from column chromatography and the individual polar lipid components obtained by TLC were quantified by GLC analysis of their fatty acid methyl esters (14,15) using an internal standard (17:0). The results are expressed as weight % of total phospholipids, total glycolipids or total neutral lipids.

RESULTS AND DISCUSSION

The various solvents and conditions used here for lipid extraction have been shown to be adequate for lipid extraction from other cereals (14-18). Since lipid solvents also extract varying amounts of non-lipid impurities, all extracts were purified by the Bligh and Dyer partitioning procedure (11). The efficiency of several solvents at extracting millet lipids has not been investigated previously. Table 1 shows the quantitative distribution of lipid classes extracted by 8 solvent systems. Although chloroform-methanol (2:1) and hexane/ether were good solvents for extraction of neutral lipids, they gave a low yield of total lipids due to poor extraction of polar lipids. Ethanol-ether-water (2:2:1) extracted more polar lipids and also was a good solvent for neutral lipids, but still gave a low yield of total lipids. Aqueous methanol or ethanol were good for extracting phospholipids from millet seeds, but they were too polar to extract all of the neutral lipids and gave low yields of total lipids. Hot water-saturated butanol (WSB) was most efficient in extracting the polar lipids as well as neutral lipids, more so than WSB at room temperature (cold WSB), and gave the highest yield of total lipids. This is in agreement with the results reported for other cereals (8,16,18). Total lipids extracted with hot WSB accounted for 7.2% of seed dry weight and consisted of about 85% neutral lipids, 12% phospholipids and 3% glycolipids.

Fatty acid analysis of the total lipids extracted by the various solvents (Table 1) showed

[2]It should be noted that solvent systems C and D form azeotropes and systems A and B also fractionate during reflux in the Soxhlet extraction procedures.

TABLE 1

Composition of Lipids Extracted from a Single Sample of Millet Seeds by 8 Solvent Systems

Solvent system[a]	Total lipid %[b]	Lipid classes[c] g/100 g dry seed			Fatty acid composition (area %)					
		NL	GL	PL	16:0	18:0	18:1	18:2	18:3	20:0
A) n-hexane/ether (80:20)	5.39	5.30	0.05	0.04	16.2	5.2	26.5	47.8	3.0	1.3
B) methanol/water (85:15)	3.00	2.21	0.22	0.57	18.2	4.2	23.7	48.6	4.6	0.6
C) $CHCl_3$/MeOH (2:1)	6.90	6.50	0.12	0.28	16.7	5.3	26.3	47.0	3.5	1.2
D) ethanol/ether/water (2:2:1)	6.20	5.45	0.11	0.64	16.3	5.4	26.6	47.9	3.0	0.7
E) ethanol/water (80:20)	2.20	1.43	0.17	0.60	20.5	3.9	23.7	45.9	5.5	0.6
F) isopropanol and $CHCl_3$/MeOH (1:2)	5.20	4.61	0.17	0.42	15.3	5.2	26.5	48.7	3.2	1.0
G) cold water-saturated butanol	6.09	5.40	0.18	0.51	16.8	5.4	26.6	46.5	3.4	1.3
H) hot water-saturated butanol	7.19	6.16	0.19	0.84	16.2	7.4	24.7	47.4	3.5	0.8

[a]See text for extraction and purification procedure.

[b]Total purified lipid, expressed as % by weight of dry seeds.

[c]Prepared by Bio-Sil BH (100-200 mesh) silica gel column chromatography of total lipids, using chloroform to elute neutral lipids (NL), acetone to elute glycolipids (GL) and methanol to elute phospholipids (PL) (12).

TABLE 2

Fatty Acid Distribution in Major Lipid Classes of Millet[a]

Lipid fraction[b]	Fatty acids (area %)					
	16:0	18:0	18:1	18:2	18:3	Others[c]
Neutral lipids (NL)	16.7	8.2	24.7	45.7	3.2	1.4
Glycolipids (GL)	19.4	4.3	24.4	39.5	10.9	1.5
Phospholipids (PL)	29.6	2.6	26.4	37.3	4.1	trace

[a]Values are averages for 4 separate analyses of fatty acids from lipids of seeds extracted with hot WSB.

[b]Fractions were prepared as described in footnote c, Table 1.

[c]Includes 14:0, 20:0 and 22:0; trace = less than 0.5%.

that linoleic (18:2) was the predominant fatty acid in all extracts, followed by oleic (18:1) and palmitic 16:0 acids. Distinct differences in fatty acid composition were observed for the NL, GL and PL fractions (Table 2), the NL fraction having the highest and lowest contents of 18:2 and 16:0 acids, respectively, while the PL fraction had the lowest and highest contents of these acids; the GL fraction had the highest content of 18:3 acid. It should be noted that, as has been observed for other seeds (19,20), the 16:0 acid content of the lipids extracted by the various solvents increased with increasing proportions of phospholipids extracted (compare solvents, A, C, B and E, Table 1) and that this can be attributed to the high content of 16:0 in the PL fraction (Table 2).

Experience with wheat lipids has shown (16) that lysophosphatidylcholine (LPC) is the most difficult lipid to extract, and the yield of LPC was taken as indicative of the thoroughness of extraction. TLC separation of the phospholipid fractions from lipids extracted by the different solvent systems showed (Fig. 1) that LPC was well-extracted by solvent systems ethanol/water (80:20), hot WSB, cold WSB and methanol/water (85:15), but not by the other solvents. Quantitative comparison of phospholipids extracted by hot WSB and chloroform/methanol (2:1) showed that LPC was the major phospholipid (42% of total) extracted with hot WSB, while less than 1% was extracted with chloroform/methanol (Table 3). In contrast, on charred TLC plates (Fig. 1) the phosphatidylcholine (PC) spot was the most intense, rather than the LPC spot. Other workers (5,8,21) also have observed PC to give a more intense spot than LPC on charred TLC plates and concluded

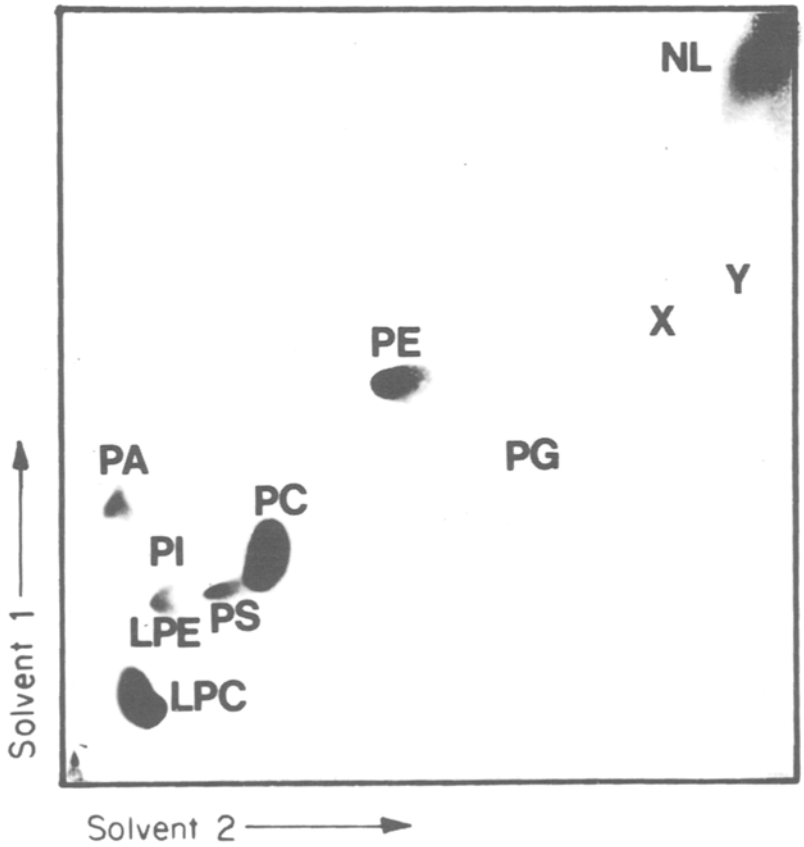

FIG. 1. Thin layer chromatographic separation of millet seed phospholipid fraction (in solvent system VI) from lipids extracted by 8 solvent systems: lane 1, ethanol/water (80:20); lane 2, $CHCl_3$/MeOH (2:1); lane 3, hot WSB; lane 4, n-hexane/ether (80:20); lane 5, cold WSB; lane 6, ethanol/diethylether/water (2:2:1); lane 7, isopropanol + $CHCl_3$:MeOH (1:2); and lane 8, methanol/H_2O (85:15). Identity of spots: X_1, unidentified; LPC, lysophosphatidylcholine; LPE (+PS), lysophosphatidylethanolamine (+phosphatidylserine); PC (+PI), phosphatidylcholine (+phosphatidylinositol); PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PA, phosphatidic acid; X_2, X_3, unidentified; and X_4, tentatively, bis-phosphatidic acid; NL, neutral lipid. Total phospholipids were separated from the total lipids by silicic acid chromatography (see footnote c, Table 1).

erroneously that PC is the major phospholipid. The explanation for this anomaly lies in the fact that LPC contains one mole of fatty acid/mole LPC with a high proportion of saturated fatty acids, while PC contains 2 moles of fatty acid/mole PC with a high proportion of unsaturated fatty acids. Since the latter are oxidized more readily than saturated acids, the PC components will char more intensely than LPC. PC, in fact, was the second predominant phospholipid, accounting for about 24% of the total phospholipids (Table 3). Also unusual was the high content of lysophosphatidylethanolamine (LPE) (21%), higher than that of phosphatidylethanolamine (PE). Phosphatidylglycerol (PG), phosphatidic acid (PA) and the presumed bis-phosphatidic acid (bis-PA, X_4) were present in low amounts (ca 1%) (Table 3). Traces of phosphatidylserine (PS) and phosphatidylinositol (PI) were present in the LPE and PC spots, respectively (Fig. 1, Table 3), as confirmed by two-dimensional TLC (Fig. 2).

The high content of lysophosphatidylcholine probably is not due to the action of phospholipase A_2 activity during the extraction procedure since the highest proportions of LPC were obtained with hot WSB, which is the best phospholipase inactivating solvent system of the solvents tested. Phospholipase activity during ripening or drying of the seeds also probably is

TABLE 3

Composition of Phosphoglycerides (% by wt) Extracted from Millet Seeds by 2 Solvent Systems[a]

Phospholipid	Hot WSB	$CHCl_3$ MeOH (2:1)
Lysophosphatidylcholine (LPC)	41.6	0.8
Lysophosphatidylethanolamine (LPE)[b]	21.2	16.0
Phosphatidylcholine (PC)[c]	24.3	62.4
Phosphatidylethanolamine (PE)	6.4	17.2
Phosphatidylglycerol (PG)	1.1	1.3
Phosphatidic acid (PA)	1.2	0.7
Biphosphatidic acid (X_4)	1.2	1.5

[a]Total phospholipids, obtained by silica gel column chromatography (see Table 1), were separated by preparative TLC in solvent system VI; the individual phospholipid components were eluted from the silica with the Bligh-Dyer solvent system (11,12) and quantitated as FAME (14,15) using an internal standard (17:0).

[b]Includes phosphatidylserine.

[c]Includes phosphatidylinositol.

TABLE 4

Fatty Acid Composition of Millet Seed Phospholipids[a]

	Fatty acids (area %)					
Phospholipid	16:0	18:0	18:1	18:2	18:3	Others[b]
LPC	46.0	3.2	28.9	18.3	3.7	trace
LPE (+PS)	41.8	2.4	20.2	33.0	2.6	trace
PC (+PI)	18.8	2.8	28.2	48.1	2.2	trace
PE	20.0	2.3	21.9	52.4	1.8	1.6
PG	8.2	2.4	10.5	70.4	1.8	6.6
PA	26.8	4.8	21.4	37.3	1.9	7.9
X_4	31.8	6.1	20.4	36.0	trace	5.7

[a]Total lipids were extracted with hot WSB and fractionated by silicic acid column chromatography (see Tables 1 and 2) and the PL fraction was separated by TLC (see Table 3); X_4 is tentatively identified as bis-phosphatidic acid.

[b]Includes 14:0, 20:0 and 22:0; trace=less than 0.5%.

not responsible for the high LPC content, since lipid degradative changes are detected only after the grain has been ground into a meal or allowed to ferment (1). LPC and other lysophospholipids are normal constituents of seeds of cereals where they exist as amylase inclusion complexes within the starch granule; these complexes promote helix formation of the newly synthesized amylase during seed development (22).

Fatty acid analysis of the individual phospholipids (Table 4) showed, as expected, high proportions of 16:0 acid in the lysophospholipids LPC and LPE which were more than twice that in the diacyl phosphoglycerides. High proportions of 18:2 acid were found in PC, PE and PG, while lower proportions of 18:1 and only small amounts of 18:3 acids were present in all of the components.

Seit-Ablaeva et al. (23) have reported the phospholipid composition of millet seeds as 13-20% PA, 12-14% PS and 18-22% PI. The proportions of these components appear to be very high compared to the values found here (Table 3). Two-dimensional TLC separation of our millet seed phospholipids (Fig. 2) showed good resolution of the minor components PS, PI, PA and PG. These results were similar to those published by Prudthi and Bhatia (8) and also to those obtained for wheat and maize (16,24). Seit-Ablaeva et al. (23) may have wrongly identified these phospholipids because of incomplete resolution on one-dimensional TLC. Phosphatidylinositol could not be resolved from PC in 3 one-dimensional TLC solvent systems that we tried; and PS and LPE could be

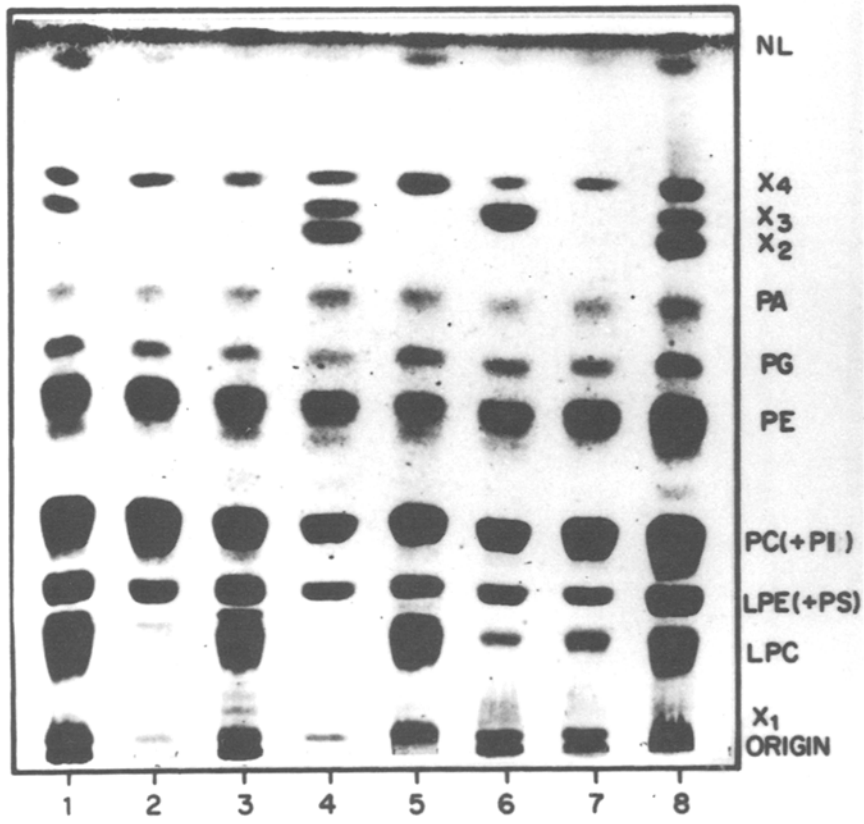

FIG. 2. Two-dimensional TLC separation of millet seed phospholipid fraction in: 1) $CHCl_3$/MeOH/H_2O (65:25:4, v/v/v); and 2) $CHCl_3$/MeOH/conc Ammonia (65:35:5, v/v/v); abbreviations as in Fig. 1; X, Y, unidentified.

TABLE 5

Composition of Millet Seed Glycolipids[a]

Glycolipid	wt %	Fatty acid composition (area %)				
		16:0	18:0	18:1	18:2	18:3
Acyl-monogalactosyldiacylglycerol	2.0	27.5	8.0	22.1	36.2	6.2
Esterified sterolglycoside	37.4	27.9	4.4	26.9	37.3	3.4
Monogalactosyldiacylglycerol	11.7	20.2	5.9	26.0	34.3	13.7
Unidentified	4.5	21.0	10.4	23.0	35.5	10.1
Sterol glycoside	14.2					
Cerebroside I	9.4	32.5	11.9	23.1	23.2	9.3
Cerebroside II	1.7	25.9	18.0	19.9	29.2	7.0
Monogalactosylmonoacylglycerol[b]	1.4	20.9	8.1	22.5	42.1	6.3
Digalactosyldiacylglycerol	15.4	16.6	5.6	20.4	38.6	18.7
Digalactosylmonoacylglycerol[b]	2.3	13.9	10.8	29.8	35.0	10.5

[a]Total lipids extracted by hot WSB were fractionated on a column of silicic acid (Table 1) and the glycolipid fraction was separated by preparative TLC in solvent system V.

[b]Identified by comparison of R_f values in solvent systems III, IV and V and in $CHCl_3$/MeOH/Acetic Acid/H_2O (65:25:8:4, v/v/v/v) with those of authentic standards.

TABLE 6

Composition of Millet Seed Non-Polar Lipids[a]

Lipid component	wt %	Fatty acid composition (area %)					
		16:0	18:0	18:1	18:2	18:3	Others[b]
Steryl ester	3.5	18.0	8.7	30.8	42.5	trace	–
Triacylglycerols	85.8	15.9	5.3	28.8	46.2	2.5	0.8
Mono- and di-acylglycerols	3.2	24.1	4.8	22.7	40.8	7.6	trace
Free fatty acids	2.1	21.1	8.8	27.2	37.8	4.1	0.8
Free sterols	5.4	–	–	–	–	–	–

[a]Total lipids were extracted by hot WSB and fractionated by silicic acid column chromatography (see Table 1). Non-polar lipids were separated by TLC on silica gel G using solvent systems I and II in the same direction.

[b]Includes 14:0, 20:0 and 22:0; trace = less than 0.5%.

completely resolved in only one solvent system (solvent III). Also, PC was inseparable from digalactosyldiacylglycerol (DGDG) when total lipid extracts were separated on TLC. It is, therefore, essential to do a preliminary fractionation of the total lipids by column chromatography in order to effectively separate and identify minor phospholipids such as PA, PS and PI in millet.

The glycolipid fraction was resolved into 10 components by TLC in solvent V (Table 5). These components were identified by co-chromatography with authentic samples and identification of the deacylated products (12). The major glycolipids were DGDG, monogalactosyldiacylglycerol (MGDG), sterol glycoside (SG) and esterified sterolglycoside (ESG). Lai and Variano-Marston (1) were unable to detect MGDG in their millet lipid extracts. In contrast to the glycolipid composition in wheat (16), sterol glycosides greatly predominate over glycosyldiacylglycerols (Table 5). Obara and Kihara (25) reported a 13.5% lipid content, containing 17.5% glycolipids, in Italian millet *(Setaria italica)*. The sugar in MGDG and DGDG was exclusively glucose, whereas it is almost entirely galactose in other cereals. These high glycolipid contents are questionable because of the difficulties in analyzing millet glycolipids by TLC of total lipid extracts. Again, preliminary fractionation of total lipids by column chromatography is recommended. In the present study, analysis has shown that the glycolipids constitute less than 8% of the total lipid extract with SG and ESG as the predominant components. The presence of small amounts of acyl-MGDG, monogalactosylmonoacylglycerol (MGMG) and digalactosylmonoacylglycerol (DGMG) in millet lipids have been demonstrated for the first time (Table 5). The cerebrosides usually were resolved into 2 spots on TLC. One of the spots co-chromatographed with authentic ceramide monohexoside in 3 solvent systems.

In regard to the neutral (non-polar) lipid fraction, triacylglycerol was found to be the major component, followed by free and esterified sterols (Table 6). The fatty acid composition of the neutral lipids (Table 2) largely reflects that of the triacylglycerols (Table 6). The free sterols and the sterols in the various "bound" sterol lipids (sterol ester (SE), SG and ESG) were isolated, after hydrolysis with methanolic-HCl (12), by preparative TLC (12, 26) and analyzed by GLC and by combined GLC-mass spectrometry (26). The same sterol composition was found for both the free and "bound" sterols, namely, campesterol (3%), unidentified 1 (21%), unidentified 2 (11%) and β-sitosterol (65%). The 2 unidentified sterols probably are not artifacts produced during methanolic-HCl hydrolysis of the "bound" sterols as was found in diatoms (26), since they were present in the "free" sterol fraction that had not been treated with methanolic-HCl.

In summary, the lipids found in millet are similar to those that have been identified previously in other cereals (13,19). The present work presents a more complete analysis of the lipid components of millet seed. This study will serve as a basis for further investigation of the metabolism of lipids during germination of millet seeds and to assess the importance of lipids in foods prepared from millet.

ACKNOWLEDGMENTS

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REFERENCES

1. Lai, C.C., and Varriano-Marston, E. (1980) Cereal

Chem. 57, 275-277.
2. Carnovale, E.E., and Quaglia, G.B. (1973) Ann. Technol. Agric. 22, 371-380.
3. Zeurcher, C.H. (1971) Monatsschr. Brau 24, 276-284.
4. Ahuja, K.L., Sekhon, K.S., and Sehgal, K.L. (1979) J. Fd. Sci. Technol. (Mysore) 16, 32-33.
5. Lai, C.C., and Varriano-Marston, E. (1980) Cereal Chem. 57, 271-274.
6. Rooney, L.W. (1978) Cereal Chem. 55, 584-590.
7. Sharma, K.P., and Goswami, A.K. (1969) J. Nutr. Diet. 6, 316-318.
8. Prudthi, I.D., and Bhatia, I.S. (1970) J. Sci. Fd. Agric. 21, 419-422.
9. Badi, S.M., Hoseney, R.C., and Casady, A.J. (1976) Cereal Chem. 53, 478-487.
10. Association of Official Agricultural Chemists (AOAC) (1975) Official Methods of Analysis, Washington DC, USA.
11. Bligh, E.G., and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911-917.
12. Kates, M. (1972) Techniques of Lipidology, pp. 398-401, North Holland/American Elsevier, Amsterdam, New York.
13. Clayton, T.A., MacMurray, T.A., and Morrison, W.R. (1970) J. Chromatogr. 47, 277-281.
14. Morrison, W.R., Mann, D.L., Soon, W., and Coventry, A.M. (1975) J. Sci. Fd. Agric. 26, 507-521.
15. Morrison, W.R., Tan, S.L., and Hargin, K.D. (1980) J. Sci. Fd. Agric. 31, 329-340.
16. MacMurray, T.A., and Morrison, W.R. (1970) J. Sci. Fd. Agric. 21, 520-528.
17. de la Roche, I.A., Andrews, C.J., and Kates, M. (1973) Plant Physiol. 51, 468-473.
18. Sahasrabudhe, M.R. (1979) J. Am. Oil Chem. Soc. 56, 80-84.
19. de la Roche, I.A., Burrows, V.D., and McKenzie, E.I.H. (1977) Crop Sci. 17, 145-148.
20. Welch, R.W. (1975) J. Sci. Fd. Agric. 26, 429-435.
21. Morrison, W.R. (1978) in Advances in Cereal Science and Technology (Y. Pomeranz, ed.) Vol. 2, pp. 221-348, American Association of Cereal Chemists, St. Paul, Minnesota.
22. Baisted, D.J. (1979) Phytochemistry 18, 1293-1296.
23. Seit-Ablaeva, S.K., Nechaev, A.P., and Denisenko, Y.I. (1973) Vop. Pitan. 4, 75-76.
24. Tan, S.L., and Morrison, W.R. (1979) J. Am. Oil Chem. Soc. 56, 531-536.
25. Obara, T., and Kihara, H. (1973) Nippon Nogei Kagaku Haishi 47, 231-236.
26. Kates, M., Tremblay, P.-A., and Volcani, B.E. (1978) Lipids 13, 34-41.

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METHODS

A Modified Radiometric Assay for 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase

NAFSIKA H. GEORGOPAPADAKOU*,[1] and BARBARA A. DIX[1], *The Squibb Institute for Medical Research, Princeton, New Jersey 08540*

ABSTRACT

A radiometric assay for measuring the activity of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase is described. The assay is based on the separation of the mevalonate product from HMG-CoA by high-voltage electrophoresis. This method is more sensitive and more specific than the NADPH-based spectrophotometric assay, and less tedious than available radiometric assays. It has been used to measure HMG-CoA reductase activity in crude extracts of *Saccharomyces cerevisiae* and in human skin fibroblasts.
Lipids 19:966-970, 1984.

INTRODUCTION

The enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase [mevalonate: $NADP^+$ oxidoreductase (acetylating CoA), EC 1.1.1.34] catalyzes the reduction of HMG-CoA by NADPH to produce mevalonic acid (MVA) (Fig. 1). The reaction is the first exclusive to isoprenoid biosynthesis and, at least in sterol biosynthesis, is the rate-limiting step (1-3). The mevalonate product is thus a key intermediate in the biosynthesis of sterols, as well as dolichol, ubiquinone and isopentenyladenosine (3,4). HMG-CoA reductase is a highly regulated enzyme; regulation occurs through changes in the rates of synthesis and degradation (5,8), and possibly through phosphorylation-dephosphorylation (9-11). The enzyme from several sources has been purified and studied (12-15), and its regulation recently has been the focus of intense research interest (16,17).

Several methods for measuring activity of HMG-CoA reductase exist. They are based on the disappearance of NADPH (18,19), the incorporation of radiolabel from HMG-CoA into MVA (20-22), or the release of CoA (23). Of these assays, the radiometric is the most sensitive and specific and has been widely used with crude enzyme preparations (24,12); it employs [^{14}C]HMG-CoA as substrate, with [^{3}H]MVA being added at the end of the incubation as an internal standard. MVA is isolated as mevalonolactone (MVAL) by thin layer chromatography (TLC). Drawbacks of the method are that it involves several manipulations and is relatively time-consuming. Most importantly, the [^{3}H]MVA standard may interfere with the measurements of [^{14}C]MVAL product at low enzyme activities.

*To whom correspondence should be addressed.

[1]Currently with Hoffman-La Roche, Inc., Nutley, NJ 07110.

In this paper a variation of the radiometric assay is described in which the separation of MVA from other components is performed by high-voltage paper electrophoresis. Percentage conversion is then calculated, total recovered radioactivity serving as an internal standard. The method involves minimal manipulations and has been applied successfully to studies with HMG-CoA reductase from yeast and mammalian sources.

MATERIALS AND METHODS

DL-[3-^{14}C]HMG-CoA (51 μCi/μmol) and Aquasol scintillation fluid were obtained from New England Nuclear Corp. (Boston, Massachusetts); DL-[3-^{14}C]MVAL (10 μCi/μmol) from Amersham Corp. (Arlington Heights, Illinois); NADPH, dithiothreitol (DTT), phenylmethanesulfonyl fluoride (PMSF), β-glucuronidase and Trizma base from Sigma Chemical Co. (St. Louis, Missouri) and Whatman 3MM paper from Fisher Scientific (Springfield, New Jersey). Tissue culture media supplies were purchased from GIBCO Laboratories (Grand Island, New York). Mevinolin was a gift from Merck, Sharp and Dohme (Rahway, New Jersey).

Yeast Culture

S. cerevisiae ATCC 9763 was grown with shaking at 30 C in YEPD medium (1% yeast extract, 2% peptone, 2% dextrose) until late log phase (OD_{660}=1.3-1.5). Cells were harvested by centrifugation, washed with 10 mM potassium phosphate buffer, pH 7.0 and, if not used immediately, stored at -20 C.

Fibroblast Culture

Cultured human fibroblasts derived from neonatal foreskin were grown as a monolayer and used between the 10th and 30th passage.

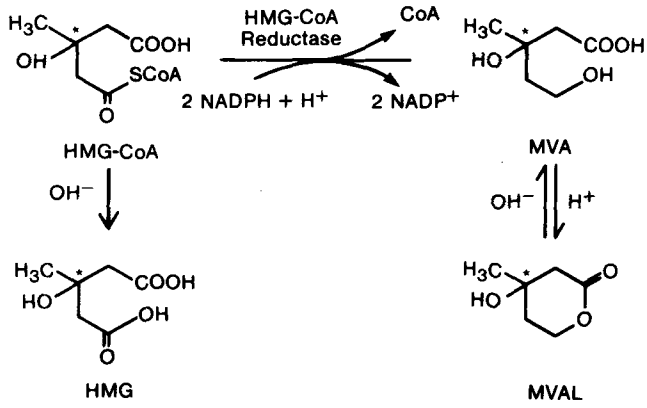

FIG. 1. Enzymatic and non-enzymatic reactions occurring in assays of HMG-CoA reductase with crude extracts. Position of the radioactive label is shown by an asterisk (*).

Stock cultures were maintained in a humidified incubator (5% CO_2) at 37 C in 250 ml (75-cm^2) flasks containing 15 ml of Eagle's minimum essential medium (GIBCO No. 3330-1435) supplemented with 0.2% $NaHCO_3$, 2 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, and 10% fetal calf serum (EMEM-FCS medium). Cells were split (1:3 ratio) every 7 days.

On day 5 the EMEM-FCS medium was replaced with EMEM, and on day 6 (20 hr prior to each experiment) with EMEM medium containing 0.01 mM mevinolin (6). On day 7 cells were dissociated with 0.025% trypsin-0.02% EDTA, harvested by centrifugation, washed with 50 mM Tris-HCl, pH 7.5 - 0.15 M NaCl, and used immediately.

Preparation of Extracts

Yeast spheroplasts were prepared by EDTA-β-glucuronidase treatment as described (25). They were lysed in 50 mM Tris-HCl, pH 7.5-2 mM $MgCl_2$, and membranes were solubilized in 50 mM potassium phosphate, pH 7.0 - 0.5% Triton X-100 at 0 C for 60 min (1). After centrifugation at 37,000 g for 30 min, the supernatant was dialyzed overnight against 100 mM potassium phosphate, pH 7.0 containing 1mM EDTA and 1 mM β-merceptoethanol. The dialysate was stored at -20 C in 0.5 ml aliquots, to avoid repeated freezing and thawing; under this condition the enzyme is stable for at least one month.

Enzyme from human fibroblast pellets was prepared by briefly homogenizing the cells (2×10^7/ml) in 50 mM Tris-HCl, pH 7.5, containing 10 mM DTT, 1 mM PMSF, and 0.5% Triton X-100, and then incubating the suspension at 0 C for 60 min (26). After centrifugation at 37,000 g for 30 min, the supernatant was stored in 0.5 ml aliquots at -20 C, where it has a half-life of 2 weeks.

Enzyme Assays

The yeast enzyme was assayed at 30 C with a 30 min incubation period. Incubation mixtures contained a total volume of 25 μl (6 × 50 mm tubes); 7.5 mM NADPH, 50 mM DTT, 0.1 mM [^{14}C]HMG-CoA (200,000 cpm), and enzyme sufficient to convert 10-13% of substrate to product. Typically, 10 μl of crude extract was used. This corresponds to 2-3 μg of protein, measured by the method of Lowry (27). The mammalian enzyme was assayed at 37 C using 2 hr incubation period. Incubation mixtures contained a total volume of 25 μl: 7.5 mM NADPH, 100 mM DTT, 0.1 mM [^{14}C]HMG-CoA and 10 μl (20-30 μg protein) of crude extract. A reaction mixture without enzyme also was run in parallel and served as a blank. Reactions were started by the addition of enzyme and were stopped by the addition of 5 μl of 10% tricholoroacetic acid (TCA).

Acidified incubation mixtures were spotted on 57 × 46 cm Whatman 3MM paper (middle of long dimension, 3 cm apart) which then was subjected to high-voltage electrophoresis (100 V/cm) at pH 3.5 (pyridine/acetic acid/water, 1/10/1000, v/v/v) for 20 min. Substrate and product (Fig. 1) were localized by autoradiography on Kodak XAR 5 X-ray film (overnight exposure). Under these conditions, the following mobilities were observed: MVAL, -1 cm; MVA, +2cm; HMG, +5.5 cm; HMG-CoA, +10 cm. The radioactive spots were cut, counted in 5 ml Aquasol, and percentage conversion of substrate (HMG + HMG-CoA + MVA + MVAL) to product (MVA + MVAL) was determined. Enzyme activity was calculated in pmoles of mevalonate

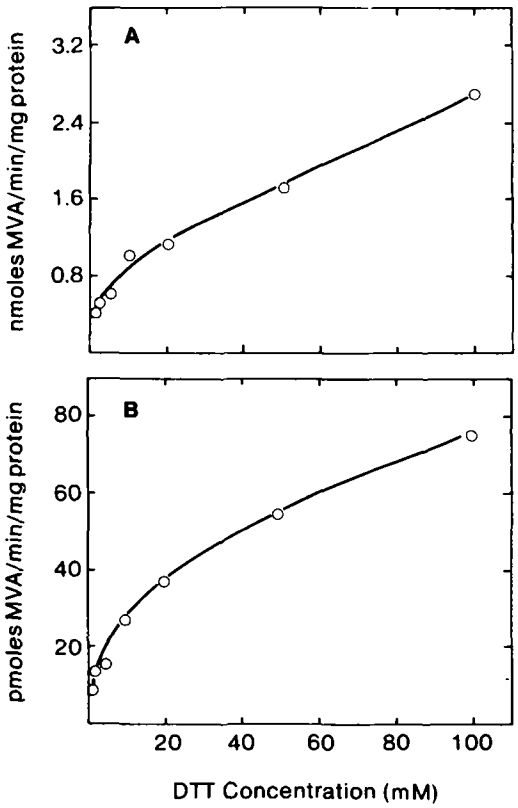

FIG. 2. Effects of DTT on HMG-CoA reductase activity in crude extracts from *S. cerevisiae* (A) and human fibroblasts (B).

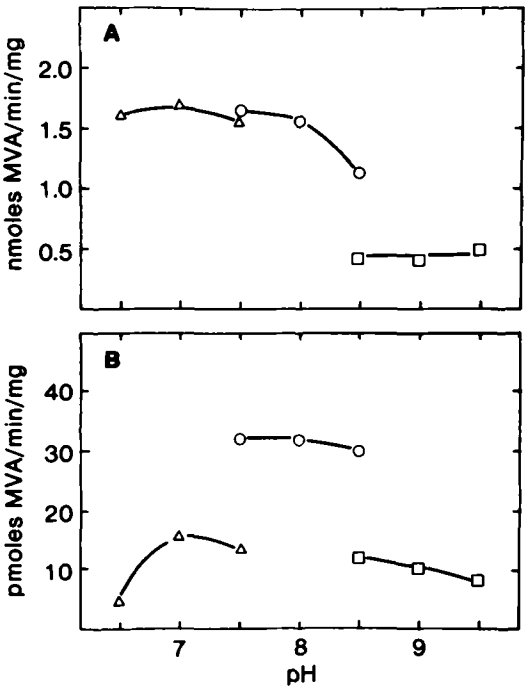

FIG. 3. pH profile of HMG-CoA reductase activity in *S. cerevisiae* (A) and human fibroblasts (B). Crude extracts were prepared as described in Methods, with 50 mM potassium phosphate (△ - △), Tris-HCl (○-○), or sodium borate (□ - □). Incubation mixtures thus contained 20 mM of the indicated buffer and pH.

formed per min per mg protein, since the reaction is linear with time and protein concentration under these experimental conditions.

RESULTS

Four major radioactive spots were detected on the electrophoretograms at the end of the enzyme incubations with [^{14}C]HMG-CoA. Of these, 2 with mobilities +5.5 and +10 cm also appeared in the incubation without enzyme. They were identified as HMG and HMG-CoA respectively, by subjecting [^{14}C]HMG-CoA to electrophoresis before and after saponification with 1 M NaOH (30 min at 40 C). The former condition gave predominantly the +10 cm spot and the latter the +5.5 cm spot. The other 2 spots, with mobilities -1 cm and +2 cm, were identified as MVAL and MVA respectively, by subjecting commercial [^{14}C]MVAL to electrophoresis after treatment with 2% TCA or 0.5 M NaOH (15 min at 30 C). The former treatment gave predominantly (85%) the -1 cm spot and the latter (98%) the +2 cm spot. Average recovery of radioactivity was 70% of original samples. Variation between duplicate assays was approx. 5%.

In preliminary experiments, the [^{14}C]MVA was incubated with crude extracts from either cell source under conditions favoring formation of MVA (pyro)phosphate (28,29). No product other than MVA and MVAL could be detected in the electrophoretogram. Mevinolin, a potent inhibitor of HMG-CoA reductase (30), completely inhibited [^{14}C]MVA formation from [^{14}C]HMG-CoA at 10 μM. Finally, using an NADPH-generating system instead of NADPH in the incubation mixture (31) gave the same activity. NADPH was used routinely in the assays in the interest of convenience.

Next, the assay variables were optimized. NADPH, between 2 and 15 mM, produced maximal activity, and 7.5 mM was used routinely. DTT substantially increased activity of both the mammalian and yeast enzymes (Fig. 2). 50 mM DTT was thus employed for assay of the yeast enzyme and 100 mM DTT for assay of the mammalian one. Both pH and the nature of the buffer affected enzyme activity (Fig. 3), the optimal pH being 7.5. Sodium borate inhibited enzyme activity in both species. Potassium phos-

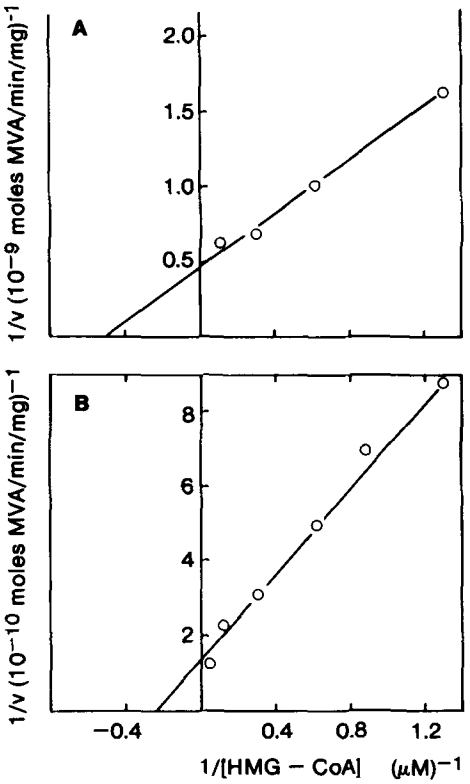

FIG. 4. Lineweaver-Burk (double reciprocal) plots of HMG-CoA reductase activity vs. HMG-CoA substrate concentration in crude extracts from *S. cerevisiae* (A) and human fibroblasts (B).

phate, Bicine (pH 8.0 and 8.5) and Hepes (pH 7.0 and 7.5) buffers inhibited activity only in human fibroblasts (data not shown). Thus, the yeast extract was routinely prepared and assayed in potassium phosphate, pH 7.5, and the fibroblast extract in Tris-HCl, pH 7.5. Preincubation of either enzyme with *Escherichia coli* phosphatase under conditions reported to convert the enzyme to the active, dephosphorylated form (31) did not affect activity. Therefore, a preincubation step was not included in the standard assay.

The K_m values for HMG-CoA in the 2 systems were 1.8 μM for the yeast enzyme and 4.2 μM for the mammalian enzyme (Fig. 4). The specific activities of the crude enzyme preparations used were 2.0 and 0.07 nmoles/min/mg protein, respectively.

DISCUSSION

A radiometric method for assaying HMG-CoA reductase has been described. Its advantages over the spectrophotometric method are sensitivity and specificity. For example, we were unable to measure mevinolin-sensitive-HMG-CoA reductase in crude extracts using the spectrophotometric assay. Presumably, other NADPH-utilizing enzymes also were present. It is noted that in human fibroblasts the small amounts of cell material and the lability of the enzyme make measurements in crude extracts not only desirable but necessary. The advantages of the present method over available radiometric assays, in particular the widely used TLC method (21,22), are 2: manipulations are kept to a minimum (protein removal and extraction with organic solvents are eliminated) and corrections are built in for incomplete recovery. Enzyme activities are initially calculated as percentage conversion of [^{14}C]HMG-CoA to [^{14}C]MVA, and subsequently are expressed as nmoles MVA/incubation. As with all radiometric assays, the present assay is a fixed-time assay. However, it may be used in kinetic studies if aliquots of the reaction mixture are removed with time, provided the reaction stays linear with assay conditions.

The K_m values for HMG-CoA obtained for the yeast and mammalian enzymes are in good agreement with those reported in the literature using other assays. For the *S. cerevisiae* the reported value is 1.2 μM (19), while for the mammalian enzyme reported values range from 0.5 to 35 μM (14, 32-36).

The specific activities obtained in cell-free extracts from the 2 species are also in line with values reported in the literature. These range from 0.24 to 2 nmoles MVA/min/mg protein for the yeast enzyme (12,37) and from 0.09 to 2.8 nmoles/min/mg protein for the mammalian enzyme (14,34,36,38). It is noted that in yeast, HMG-CoA reductase activity increases with the age of the culture, being maximal during stationary phase (1). We routinely used late log-phase cells to facilitate spheroplast formation. With fibroblasts, the observed tenfold increase in activity after mevinolin treatment is comparable to the reported increase after treatment with the structurally related compactin (6). In hepatocytes, however, mevinolin has been reported to repress HMG-CoA reductase (39).

In conclusion, the present assay is sensitive, accurate and easy to perform. It is thus well-suited for studies on the regulation of HMG-CoA reductase and its role in the biosynthesis of sterols and other important isoprenoids.

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REFERENCES

1. Quain, D.E., and Haslam, J.M. (1979) J. Gen. Microb. 111, 343-351.
2. Siperstein, M.D., and Fagan, V.M. (1966) J. Biol. Chem 241, 602-609.
3. Rodwell, V.W., Nordstrom, J.L., and Mitschelen, J.J. (1976) Adv. Lipid Res. 14, 1-74.
4. Faust, J.R., Goldstein, J.L., and Brown, M.S. (1979) Arch. Biochem. Biophys. 192, 86-99.
5. Boll, M., Lowel, M., Still, J., and Bendt, J. (1975) Eur. J. Biochem. 54, 435-444.
6. Brown, M.S., Faust, J.R., Goldstein, J.L., Kaneko, I., and Endo, A. (1978) J. Biol. Chem. 253, 1121-1128.
7. Rine, J., Hansen, W., Hardeman, E., and David, R.W. (1983) Proc. Natl. Acad. Sci. USA 80, 6750-6754.
8. Tanaka, R.D., Edwards, E.A., Lan, S.F., and Fogelman, A.M. (1983) J. Biol. Chem. 258, 13331-13339.
9. Beg, Z.H., Stonik, J.A., and Brewer, H.B. (1979) Proc. Natl. Acad. Sci. USA 76, 4375-4379.
10. Gibson, D.M., and Ingebritsen, T.S. (1978) Life Sci. 23, 2649-2664.
11. Beg, Z.H., and Brewer, H.B. (1982) Fed. Proc. 41, 34-38.
12. Qureshi, N., Dugan, R.E., Nimmannit, S., Wu, W.H., and Porter, J.W. (1976) Biochemistry 15, 4184-4190.
13. Bensch, W.R., and Rodwell, V.W. (1970) J. Biol. Chem. 245, 3755-3762.
14. Ness, G.C., Spindler, C.D., and Moffler, M.H. (1979) Arch. Biochem. Biophys. 197, 493-499.
15. Beg, Z.H., Stonik, J.A., and Brewer, H.B. (1977) FEBS Lett. 80, 123-129.
16. Chin, D.J., Luskey, K.L., Faust, J.R., MacDonald, R.J., Brown, M.S., and Goldstein, J.L. (1982) Proc. Natl. Acad. Sci. USA 79, 7704-7708.
17. Hardeman, E.C., Jenke, H.S., and Simoni, R.D. (1983) Proc. Natl. Acad. Sci. USA 80, 1516-1520.
18. Fimognari, G.M., and Rodwell, V.W. (1965) Biochemistry 4, 2086-2090.
19. Kirtley, M.E., and Rudney, H. (1967) Biochemistry 6, 230-238.
20. Goldfard, S., and Pitot, H.C. (1971) J. Lipid Res. 12, 512-515.
21. Neprokoeff, C.M., Lakshmanan, M.R., Ness, G.C., Dugan, R.E., and Porter, J.W. (1974) Arch. Biochem. Biophys. 160, 387-393.
22. Shapiro, D.J., Nordstrom, J.L., Mitschelen, J.J., Rodwell, V.W., and Schimke, R.T. (1974) Biochim. Biophys Acta 370, 369-377.
23. Hulcher, F.H., and Oleson, W.H., (1973) J. Lipid Res. 14, 625-631.
24. Brown, M.S., Dana, S.E., and Goldstein, J.L. (1973) Proc. Natl. Acad. Sci. USA 70, 2162-2166.
25. Cabib, E. (1971) in Methods in Enzymology (Jacoby, W.B., ed.) Vol. 22, pp. 120-122, Academic Press, New York.
26. Kennelly, P.J., Brandt, K.G., and Rodwell, V.W. (1983) Biochemistry 22, 2784-2788.
27. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
28. Flint, A.P.F. (1970) Biochem. J. 120, 145-150.
29. Beytia, E., Dorsey, J.K., Marr, J., Cleland, W.W., and Porter, J.W. (1970) J. Biol. Chem. 245, 5450-5458.
30. Alberts, A.W., Chen, J., Karon, G., Hunt, V., Huff, J., Hoffman, C., Rothroch, J., Lopez, M., Joshua, H., Harris, E., Patchett, A., Monoghan, R., Currie, S., Stapley, E., Albers-Schonberg, G., Hensens, O., Hirschfield, J., Hoogstem, K., Liesch, J., and Springer, J. (1980) Proc. Natl. Acad. Sci. USA 77, 3957-3961.
31. Brown, M.S., Goldstein, J.L., and Dietschy, J.M. (1979) J. Biol. Chem. 254, 3144-3149.
32. Srinkantaiah, M.V., Tormanen, C.D., Redd, W.L., Hardgrave, J.E., and Scallen, T.J. (1977) J. Biol. Chem. 252, 6145-6150.
33. Langdon, R.B., and Counsell, R.E. (1976) J. Biol. Chem. 251, 5820-5823.
34. Kawachi, T., and Rudney, H. (1970) Biochemistry 9, 1700-1705.
35. Edwards, P.A., Lemangello, D., and Fogelman, A.M. (1979) J.Lipid Res. 20, 40-46.
36. Edwards, P.A., Lemangello, D., and Fogelman, A.M. (1979) Biochim. Biophys. Acta 574, 123-135.
37. Berg, D., Draber, W., von Hugo, H., Hummel, W., and Mayer, D. (1981) Z. Naturforsch. 36c, 798-803.
38. Kleinsek, D.A., and Porter, J.W. (1979) J. Biol. Chem. 254, 7591-7599.
39. Edwards, P.A., Lan, S.-F., Tanaka, R.D., and Fogelman, A.M. (1983) J. Biol. Chem. 258, 7272-7275.

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Mass Spectrometric Location of Double Bonds in Unsaturated Fatty Acids Including Conjugated Acids as Their Methoxybromo Derivatives

N.C. SHANTHA and **T.N.B. KAIMAL,*** *Regional Research Laboratory (CSIR), Hyderabad 500 007, India*

ABSTRACT

Methoxybromo derivatives of unsaturated fatty acids including conjugated acids yield simple mass spectra and can be used to locate the position of double bonds in these acids. The derivatives are prepared under mild conditions by bromination of the unsaturated fatty acids in methanol. The method is illustrated with the methoxybromo derivatives from methyl esters of oleic, petroselenic, erucic, undecenoic, linoleic, linolenic, the conjugated diene acids from dehydrated castor oil, α-eleostearic, punicic and parinaric acids. Unlike other methods using methoxy derivatives, the methoxybromo derivatives yield fewer ions, the diagnostic peaks forming the most intense ions of the spectra. While unambiguous double bond location is possible with monoenoic acids and conjugated fatty acids, only the end carbon atoms of the unsaturation system in nonconjugated polyenoic acids is located. But the characteristic appearance of fragments corresponding to $[CH_3(CH_2)_nCH(OMe)CH(Br)CH_2\text{-}2H]^+$ and fragments 24 mass units higher than those which locate the end carbons in such fatty acids indicate a methylene-interrupted system of double bonds.

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INTRODUCTION

A number of methods have been reported for the location of double bonds in monoenoic and non-conjugated polyenoic fatty acids. Owing to the easy migration of double bonds upon electron impact (1,2), most of these methods use chemical labelling of the double bond positions prior to mass spectrometry. The earlier methods have been reviewed (3,4). Recent proposals include reactions between fatty acids and vinylmethyl ether in the ion source under charge exchange conditions (CS_2/N_2) resulting in the formation of cyclobutane adducts (5); negative chemical ionization and collisionally activated dissociation and mass analyzed ion kinetic energy (CAD MIKE) spectra (6); the Diels-Alder adducts of olefins with ketals of tetrachlorocyclopentadiene (7); nicotinic acid derivatives of fatty alcohols (8), and pyrrolidides of the products of deuterodiimide reduction (9). No method exists at present for the location of double bonds in conjugated unsaturated acids.

The existing methods require cumbersome and time consuming derivatization steps often using expensive and toxic (for example, osmium tetroxide) reagents. The method reported in this investigation, applicable to both conjugated and nonconjugated acids, uses methoxybromo derivatives easily prepared under mild conditions by addition of bromine to a methanolic solution of the ester. Fragments containing methoxy groups form a simple spectrum. The drawbacks encountered with the polymethoxy derivatives are absent. There has been an attempt to prepare methoxyhalogeno derivatives by demercuration of the methoxymercuriacetate adducts with bromine or iodine (10). However, the derivatives prepared thus from diene and triene fatty acids did not yield useful mass spectra.

*To whom correspondence should be addressed.

EXPERIMENTAL

Materials

Methyl oleate was purchased from Acme Synthetic Chemicals, Bombay. Methyl undecenoate and methyl petroselenate were available in the laboratory. Methyl erucate was isolated from mustard oil, methyl linoleate from safflower oil and linolenate from linseed oil, by silver ion TLC of the total methyl esters of the respective oils. A mixture of (E,E) and (E,Z)-methyl 9,11-octadecadienoate was isolated from dehydrated castor oil methyl esters by silver ion TLC. Other conjugated acids were isolated from *Momordica charantia* (α-eleostearic acid; (Z,E,E)-methyl-9,11,13-octadecatrienoic acid), *Trichosanthes anguina* (punicic acid; (Z,E,Z)-methyl-9,11,13-octadecatrienoic acid) and from *Impatiens balsamina* (parinaric acid; (Z,E,E,Z)-methyl-9,11,13,15-octadecatetraenoic acid) by TLC of the total methyl esters on silica gel G. Each sample was checked for its purity by gas chromatography on a Silar 10C or SE-30

column for nonconjugated and conjugated acids, respectively.

METHODS

Preparation of Methoxybromo Derivatives

Bromination of the fatty acid methyl esters was carried out in methanol. About 10 mg of ester dissolved in 10 ml of methanol (analytical reagent grade) was taken in a 2-necked, round-bottomed flask provided with a gas inlet tube. The flask was cooled to 0 C and a stream of nitrogen was bubbled through the solution. A solution of bromine in methanol (30%, wt) was added drop by drop and the mixture was stirred magnetically. Addition of bromine was continued until a faint yellow color persisted; stirring was continued for another 30 min. The brominated products were isolated by extraction with ether.

The reaction products were separated by preparative TLC on silica gel G using hexane-diethyl ether (90:10, v/v) as solvent. The fractions with the highest and the lowest Rf values were rejected. While unreacted ester constituted the former, the latter is assumed to be the polybromo derivatives. The methoxybromo derivatives resolve into more than one band; for example, 3 bands are obtained for trienoic acids all of them not necessarily yielding identical mass spectra. Hence, when analyzing polyenoic acids, analysis of the various products may be necessary. Rf values of the relevant bands cannot be specified as they vary with the number of double bonds in the original ester, but generally they ranged between 0.3 and 0.6.

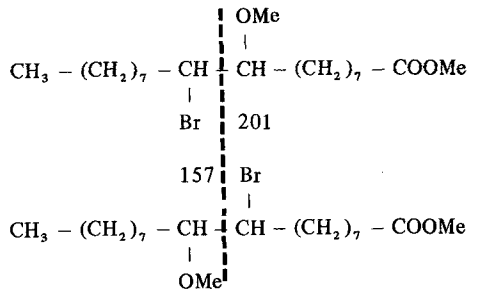
Mass Spectrometry

Mass spectra were recorded under electron impact conditions on a VG Micromass 7070 H high resolution mass spectrometer equipped with a minicomputer (Digital PDP 8A) with floppy disc recorder (RX 02) and 16K core memory. Samples were introduced by either a direct inlet system for solid samples or a septum inlet system for liquid samples. The source temperature was 180 C and emission 200 μA at 70 eV. A few spectra also were recorded at 20 eV.

RESULTS

Methyl Oleate

Bromination in methanol of methyl oleate ((Z)-methyl-9-octadecenoate) produces 2 isomeric bromomethoxy derivatives.



Cleavage between the carbons bearing the Br and OMe groups gives high intensity ions at m/z 201(100) and 157(97). The figures in parentheses indicate the relative intensity. Peaks arising from the loss of MeOH from these fragments at m/z 169(16) and 125(6) also are present. The former loses an additional molecule of MeOH to form the peak at m/z 137(20). The spectrum is simple, making the location of the double bond easy. A molecular ion peak, as could be expected, is not observed. But a pair of low intensity ions at m/z 375 and 377(3), corresponding to the acylium ion (M^+-31), and that arising from a further loss of HBr from this ion, at m/z 295(4), are seen. The fragment at m/z 263(4) should arise by the loss of MeOH from the ion m/z 295.

Methyl Petroselenate

The diagnostic peaks correspond to m/z 159 (100) and 199(77). Peaks also are present, in conformity with the observations made with methyl oleate, at m/z 375 and 377 (M^+-OCH_3) (<1) at m/z 295 (M^+-OCH_3-HBr) (8) and at m/z 263 (M^+-OCH_3-HBr-CH_3OH) (4).

Methyl Eurcate

Cleavage between 13, 14 position gives intense ions at m/z 157(100) and 257(78). These fragments lose methanol to generate ions at m/z 125(8) and 225(16), respectively. Fragments containing the carbomethoxy and the methoxy groups can lose an additional molecule of methanol, in the present case yielding the ion at m/z 193(8).

Methyl Undecenoate

The diagnostic peaks appear at m/z 215 (100) and m/z 45(97%). The peaks at m/z 183 (26) and 151(29) arise by the loss of one and 2 molecules of CH_3OH, respectively, from this fragment. The doublet at m/z 137, 139(33) arises from the fragment $[CH_2(Br)\,CH(OMe)]^+$. However, the other fragment arising by this scission, namely that of mass 171, does not

appear in the spectrum. Obviously, the charge is stabilized on the former fragment, owing to the presence of both Br and OMe. This doublet at m/z 137, 139 appears to be characteristic of terminal olefins since the corresponding fragments were not observed in any of the internal olefins studied.

Methyl Linoleate

The fragments of m/z 201(86) and 115(100) locate the end carbons of the 2 double bonds present in this acid. A doublet appears at m/z 219, 221(23), which corresponds to the fragment $[CH_3(CH_2)_4\ CH(OMe)\ CH(Br)\ CH_2\text{-}2H]^+$. Two prominent peaks 24 mass units higher than these, namely m/z 225(60) and 139(21), also appear in the spectrum.

Methyl Linolenate

The methoxybromo derivatives of methyl linolenate show intense peaks at m/z 73(100) and 201(61), locating again the end carbons of the trienoic system. Fragments arising from the methoxy groups on the carbons corresponding to the middle double bond do not appear in the spectrum. Such a fragment with 2 methoxy and bromine groups could be expected to have a very short life. As observed with linoleate, a doublet of equal intensity corresponding to $[CH_3\text{-}CH_2\text{-}CH(OMe)\text{-}CH(Br)\text{-}CH_2\text{-}2H]^+$ appears at m/z 177, 179(33). Further, fragments of mass 24 units higher than those which locate the end double bonds are present again in conformity with the observation made with linoleate. Though the genesis of these latter fragments is difficult to explain, this appears to be a characteristic feature of fatty acids with a methylene-interrupted system of double bonds.

One notable feature in the spectra of methoxybromo derivatives is the absence of ions arising by cleavage α to the methoxy group which considerably simplifies the spectra. Cleavage appears to take place only between carbons bearing the bromine and the methoxy groups, respectively. The only exception was the terminal olefinic compound, methyl undecenoate.

Conjugated Acids

Dienes. This class of compounds is exemplified by methyl-9,11-octadecadienoate. With a conjugated diene both 1,2- and 1,4-addition can take place. This, however, did not complicate the spectrum since fragments arising from the 1,4-adduct are of higher intensity than those arising from the 1,2-adduct and the double bonds are easily located (fragments at m/z 129 (30) and 201(46)). Those from the 1,2-adduct produce fragments at m/z 155(26) and 227(11).

Trienes. Methyl α-eleostearate and methyl punicate were studied under this class. Differing only in the configuration of one of the 3 double bonds, these isomers yield identical spectra. The end carbons of the conjugated system are clearly indicated by the peaks at m/z 101(56) and 201(26). While these peaks should arise from the derivatives formed by 1,6-addition, 1,4-addition (peaks at m/z 127 (38) and 227(10) and 1,2-addition (peaks at m/z 153(5) and 253(6) also take place to a lesser extent.

Tetraenes. The spectrum corresponding to the derivatives of methyl parinarate shows high intensity ions at m/z 73(100) and 201(53) arising by 1,8-addition and locates the end carbons of the conjugated system. In addition to the predominant 1,8-adduct, products arising by 1,6-addition (m/z 99(10) and 227(4)), 1,4-addition (m/z 125(7) and 253(2)) also were present, while those arising by 1,2-addition yielded low intensity ions (m/z 151 and 279, <1).

As observed with monoenoic and nonconjugated polyenoic acids, alpha cleavage is virtually absent, though in these cases the Br and OMe groups are not on adjacent carbons, excepting the 1,2-adduct. While simple methoxy derivatives give peaks corresponding to scission on either side of the carbon bearing the methoxy group, the presence of Br in the methoxybromo derivatives appears to direct the cleavage of the molecule to one side only; that is, to the side where the Br is located. Thus in the above cases cleavage between C_8 and C_9 is suppressed, and the intensity of the ion (m/z 157) arising by this cleavage is $<1\%$.

DISCUSSION

The above cited examples show the utility of the method in locating the double bonds in conjugated and nonconjugated fatty acids. While for the former class of compounds no such method exists, for the latter, the present method offers advantages over existing ones.

The derivatives are easily prepared from inexpensive and commonly available reagents under mild conditions (room temperature). This is important especially with polyenoic acids of conjugated unsaturation. The whole sequence of operations can be completed in ca. 2 hr. Low intensity of the diagnostic peaks is a problem with almost all of the existing methods. The methoxybromo derivatives on the other hand give high intensity ions, the diagnostic peaks forming the most intense peaks in the spectra. Owing to the absence of

α-cleavage, these derivatives yield simple spectra containing fewer ions compared to the derivatives reported so far. Fragments containing bromine do not appear in the spectrum except in a few cases at high mass ranges in monoenoic and conjugated polyenoic acids. In polyenoic acids with a methylene-interrupted system of double bonds, a diagnostic Br-containing fragment appears, corresponding to $[CH_3(CH_2)_n CH(OMe)CH(Br)CH_2\text{-}2H]^+$. Halogens from aliphatic halides are lost easily during mass spectrometry. McLafferty (11), who made a comprehensive study of the monohalogenated aliphatic compounds, concluded there was a scarcity of halogen containing ions in their mass spectra. This may be attributed to the high electron affinity of the Group VII elements.

The Reaction

The mode of addition of halogens to olefinic systems is well documented (12-15). Halogenation in a nucleophilic solvent such as methanol (16-18) yields a mixture of dihalides and methoxyhalogeno derivatives.

A study of the addition of bromine to hexa-1,3,5-triene led to the isolation of 1,6- and 1,2-dibromides only (19). However, the mass spectra of the methoxybromo derivatives of methyl α-eleostearate indicate the presence of isomers formed by 1,2-, 1,4- and 1,6-addition. The fragmentation pattern also indicates that the bromonium ion undergoes rearrangement so that in the final product the bromine appears on the fourth carbon, rather than the first, of the conjugated system. This is in conflict with the postulated mechanisms of electrophilic addition to conjugated systems. This aspect needs investigation.

In conclusion it may be stated that the present method satisfies the need for a reliable method for the location of double bonds in conjugated polyenoic acids. The method applies equally well to isomeric monoenoic acids. For methylene interrupted polyenoic acids the method locates only the terminal double bonds. But the characteristic presence of fragments corresponding to $[CH_3(CH_2)_n CH(OMe)CH(Br)CH_2\text{-}2H]^+$ and the fragment 24 mass units higher than those which locate the terminal carbons of the polyenoic system, clearly indicate a methylene-interrupted system of double bonds.

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REFERENCES

1. Ryhage, R., and Stenhagen, E. (1963) in Mass Spectrometry of Organic Ions, F.W. McLafferty (ed.), Academic Press, New York, p. 399.
2. McCloskey, J.A. (1970) in Topics in Lipid Chemistry, F.D. Gunstone (ed.), Logos Press, London, Vol. 1, p. 369.
3. Minnikin, D.E. (1978), Chem. Phys. Lipids 21, 313-347.
4. Dommes, V., Wirtz-Peitz, F., and Kunau, W.H. (1976), J. Chromatogr. Sci. 14, 360-365.
5. Chai, R., and Harrison, A.G. (1981), Anal. Chem. 53, 34-37.
6. Bambagiotti, M., Coran, S.A., Gianellini, V., and Vincieri, F.F. (1983), Org. Mass Spectrom. 18, 133-134.
7. Kidwell, D.A., and Biemann, K. (1982), Anal. Chem. 54, 2462-2465.
8. Vetter, W., and Meister, W. (1981) Org. Mass Spectrom. 16, 118-122.
9. Kawaguchi, A., Kobayashi, Y., Ogawa, Y., and Okuda, S. (1983), Chem. Pharm. Bull. (Japan) 31, 3228-3232.
10. Minnikin, D.E. (1975) Lipids 10, 55-56.
11. McLafferty, F.W. (1962), Anal. Chem. 34, 2-15.
12. Ingold, C.K. (1953), Structure and Mechanism in Organic Chemistry, Cornell University Press, Ithaca, New York, pp. 646-721.
13. de la Mare, P.B.D., and Bolton, R. (1966), Electrophilic Additions to Unsaturated Systems, Elsevier, New York.
14. Traylor, T.G. (1969), Accounts Chem. Res. 2, 152-160.
15. Fahey, R.C. (1968), Topics Stereochem. 3, 238-342.
16. Conant, J.B., and Jackson, E.L. (1924) J. Am. Chem. Soc. 46, 1727-1730.
17. Jackson, E.L. (1926), J. Am. Chem. Soc. 48, 2166-2174.
18. Bartlett, P.D. and Tarbell, D.S. (1936), J. Am. Chem. Soc. 58, 466-474.
19. Farmer, E.H., Laroia, B.D., Switz, T.M., and Thorpe, J.F. (1927), J. Chem. Soc. 130, 2937-2958.

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COMMUNICATIONS

Effects of Organic Solvents on Lipase for Fat Splitting

KEE H. KIM, DAE Y. KWON and **JOON S. RHEE**, Department of Biological Science and Engineering, Korea Advanced Institute of Science and Technology, P.O. Box 150, Chongyangni, Seoul, Korea

ABSTRACT

The effect of organic solvents on the stability and catalytic activity of the microbial lipase from *Candida rugosa* for hydrolysis of triglyceride (fat splitting) has been examined. The solvents examined were 5 hydrocarbons (n-hexane, n-heptane, n-octane, iso-octane and cyclohexane) and 3 ethers (diethylether, diisopropylether and di-n-butylether). The results revealed that iso-octane and cyclohexane are superior to the other solvents examined for enzymatic fat splitting in organic solvent systems.
Lipids 19:975-977, 1984.

INTRODUCTION

In recent years, the bioconversion or biotransformation of steroids and lipids in water-immiscible organic solvents has been studied intensively, because these organic systems are advantageous when steroids and lipids which are poorly soluble in water are used (1,2). In contrast to the bioconversion of steroids, however, there are a limited number of research papers on the bioconversion of lipids in organic solvents. According to Çelibi et al., the rate of lipid hydrolysis by lipase from *Candida cylindracea* (syn. *C. rugosa*) was increased appreciably when such solid lipids as tripalmitin and tristearin were dissolved in n-heptane (2). Bell et al. used the solvent diisopropylether in the hydrolysis of the trglycerides by mycelial lipase from *Rhizopus arrhizus* (3). However, they did not elaborate any rationale as to why they used those specific solvents for hydrolysis of triglycerides (fat splitting).

The objective of this report is to select the solvent most suitable for fat splitting by lipase in terms of lipase stability and catalytic activity of lipase. In this report lipase from *Candida rugosa*, which has random specificity of reaction relative to the positions and fatty acids of triglycerides (4), was used to study the hydrolysis of olive oil.

MATERIALS AND METHODS

The lipase from *Candida rugosa* was purchased from Sigma Chemical Co. (St. Louis, Missouri, USA).

The solvents used were 5 hydrocarbons (n-hexane, n-heptane, n-octane, iso-octane and cyclohexane) and 3 ethers (diethylether, diisopropylether and di-n-butylether). These solvents were purchased from Tokyo Kasei Chemical Co., Ltd. (Tokyo, Japan), and highly refined olive oil and tributyrin were specifically manufactured by Sigma as lipase substrates. All other reagents and chemicals used were of analytical grade. To maintain the constant water-solvent ratio of each solvent, all of the solvents used were saturated with water before treatments.

Effects of the solvent on the lipase were studied by measuring the lipase stability and activity in a well-stirred batch reactor. One unit of lipase activity was defined as one micromole of fatty acids produced per 20 min under the analytical conditions. The stability of the lipase in solvents was estimated by determining the residual activity of the lipase after thorough mixing with each solvent for 1, 2, 4, 6 and 8 hrs at 35 C. Lipase solution was prepared by dissolving 50 mg of lipase powder (155 units per 1 mg lipase powder, according to the supplier) in 5 ml of 0.05 M phosphate buffer (pH 6.5) at 4 C; 5 ml of the lipase solution was added to each solvent (50 ml), thermally equilibrated at 35 C, and agitated at 1,000 rpm in the stirred batch reactor. After stopping the agitation at the predetermined time intervals, 100 μl of the lipase solution were taken off from the underlayer of the mixture of solvent and enzyme by microsyringe. This enzyme solution was added to a 5% (v/v) emulsion solution of the tributyrin which contained 5% (w/v) gum arabic. The residual activity of lipase was determined by titrating the butyric acid produced with 0.05 N sodium hydroxide (5).

Lipase activity in each solvent was determined by assaying the fatty acids produced by a rapid colorimetric method (6). Phosphate buffer (5 ml, 0.05 M, pH 6.5) including 10 mg lipase powder was added to 50 ml of olive oil-solvent reaction mixture at 35 C. After incuba-

tion for 20 min in the stirred batch reactor, 10 ml of 6N-HCl was added and agitation was continued for about 30 sec. After stopping the agitation, the supernatant composed of fatty acids and solvent was taken, the solvent was evaporated from this supernatant with a rotary vacuum evaporator, and the resulting fatty acids were redissolved in n-hexane. Finally lipase activity in n-hexane was determined by observing the absorbance at 715 nm followed by computing the fatty acids produced from the calibration curve of fatty acids vs. absorbancy.

RESULTS AND DISCUSSION

One objective of using a water-immiscible solvent is to minimize the exposure of the enzyme to organic solvents. Thus the solubility of the organic solvent in water may be important, since it causes either inhibition of the reaction or inactivation of the enzyme as reviewed by Lilly (7). Based on this property, we excluded carbonyls (aldehydes and ketones) and halogenated hydrocarbons, since they have higher solubility in water. Aromatic hydrocarbons also were excluded, because they are highly toxic to human beings and because they yielded lower proportions of fatty acids according to our preliminary tests. We included only aliphatic and cyclic hydrocarbons and ethers as the possible solvent system. Besides the water solubility and toxicity of the solvents, other physicochemical properties of the solvent must be considered in selecting a solvent for enzymatic fat splitting: density, dielectric constant, boiling point, freezing point, protonic activity towards buffers, interfacial tensions, hydrophille-lipophille-balance and propensity for hydrogen bond formation (8). However, we could not pinpoint any criterion for selecting the solvent for fat splitting on the basis of these physicochemical properties.

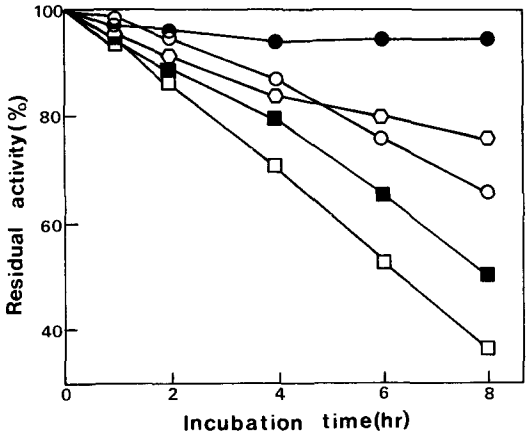

FIG. 1. Stability of lipase in hydrocarbons. After incubation of lipase with each of the solvents for 1, 2, 4, 6 and 8 hrs at 35 C, residual activity was determined by titrating the fatty acids produced in tributyrin emulsion with 0.05 N NaOH. Each point represents the mean value of 3 determinations. Symbols: ○ = n-hexane; □ = n-heptane; ● = n-octane; ■ = iso-octane, and ○= cyclohexane.

The lipases were relatively unstable in all hydrocarbons except n-octane (Fig. 1), whereas the lipases were all stable in the ether group. The residual activities in the ether group slightly decreased up to 2 hr and remained constant thereafter at about the 90-95% level. The lipases in n-octane and the ether group were, in fact, almost as stable as in the aqueous solution at

TABLE 1

Lipase Activity in Various Organic Solvents[a]

Solvent	Lipase activity for olive oil		
	3%[b]	10%	30%
Hydrocarbons			
n-Hexane	15.5 ± 0.2[c]	63.3 ± 0.3	213.3 ± 12.0
n-Heptane	14.1 ± 0.3	42.3 ± 1.3	158.7 ± 4.6
n-Octane	25.2 ± 2.2	42.3 ± 1.2	172.8 ± 6.6
Iso-octane	103.2 ± 1.1	326.2 ± 2.1	710.0 ± 31.0
Cyclohexane	64.1 ± 1.3	180.0 ± 2.5	414.0 ± 16.1
Ether			
Diethyl ether	<5.0	<5.0	15.9 ± 1.5
Diisopropyl ether	<5.0	25.2 ± 0.9	99.0 ± 1.8
Di-n-butyl ether	<5.0	<5.0	<5.0

[a]One unit is defined as one μmole of fatty acids/20 min/5 ml of enzyme solution.
[b]Olive oil concentration, % (v/v).
[c]Mean value ± SD based on 3 samples.

35 C (9). The lipase activity must be measured within the time range in which the activities of lipase do not appreciably change with the types of the solvent. The above results show that the residual activity of the lipase remained at about the 98% level when the enzyme was incubated for 20 min, regardless of the solvents used.

Table 1 shows the lipase activity in the organic solvents used at the substrate (olive oil) concentration of 3, 10 and 30% (v/v). The data indicate that the rate of the enzymatic fat hydrolysis increased with the increase of the substrate concentration up to 30% olive oil, whereas in the emulsion system the rate of fat hydrolysis was increased only up to 5% of olive oil concentration and decreased substantially thereafter with the increase of substrate concentration as shown by Kwon and Rhee (10). According to Linfield et al., the impurities in the olive oil inhibited the lipase activity (11). However, we did not find any inhibition in our study, as we used highly refined olive oil. Furthermore, branched (iso-octane) and cyclic (cyclohexane) hydrocarbons yielded much higher lipase activity than the group of straight-chain aliphatic hydrocarbons. On the other hand, the ether group yielded very poor activities despite their excellent stability. This poor activity probably is due to the competitive inhibition of ether molecules. Brockerhoff suggested, in fact, that the diethylether molecule reacts as a substrate inhibitor, thus inhibiting the activity of the lipase competitively (12).

From the above results, it can be concluded that iso-octane and cyclohexane are better solvents than any of the other solvents examined for enzymatic fat splitting in organic solvent systems.

REFERENCES

1. Cremonesi, P., Carrea, G., Ferrara, L., and Antonini, E. (1975) Biotechnol. Bioeng. 17, 1101-1108.
2. Çelibi, S.S., Uçar, T., and Çağlar, M.A. (1981) in Advances in Biotechnology, (Moo-Young, M., Robinson, C.W., and Vezina, C., eds.) Vol. 1, pp. 691-697, Pergamon Press, Toronto.
3. Bell, G., Todd, J.R., Blain, J.A., Patterson, J.D.E., and Shaw, C.E.L. (1981) Biotechnol. Bioeng. 23, 1703-1719.
4. Benzonana, G., and Esposito, S. (1971) Biochim. Biophys. Acta 231, 12-22.
5. Lieberman, R.B., and Ollis, D.F. (1975) Biotechnol. Bioeng. 17, 1401-1419.
6. Lowry, R.R., and Tinsely, I.J. (1976) JAOCS 53, 470-472.
7. Lilly, M.D. (1982) J. Chem. Tech. Biotechnol. 32, 162-169.
8. Butler, L.G. (1979) Enzyme Microb. Technol. 1, 253-259.
9. Tomizuka, N., Ota, Y., and Yamada, K. (1966) Agric. Biol. Chem. 30, 576-584.
10. Kwon, D.Y., and Rhee, J.S. (1984) Korean J. Chem. Eng. 1, 153-158.
11. Linfield, W.M., O'Brien, D.J., Serota, S., and Barauskas, R.A. (1984) JAOCS 61, 1067-1071.
12. Brockerhoff, H. (1969) Arch. Biochem. Biophys. 134, 366-371.

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Wide Variations of Plasma Triglyceride Concentrations in Guinea Pigs

N. MORI[a,b], T. MURASE[a,*], N. YAMADA[a], N. ARAKAWA[b] and F. TAKAKU[a],
[a]*The Third Department of Internal Medicine, Faculty of Medicine, University of Tokyo, Hongo, Tokyo, 113, Japan, and* [b]*Department of Food and Nutrition, Ochanomizu University, Otsuka, Tokyo, 113, Japan*

ABSTRACT

Guinea pigs have varying plasma triglyceride concentrations ranging from 28 to 1392 mg/dl, with relatively uniform plasma cholesterol and phospholipid levels. To understand why the animals exhibit such wide variations of plasma triglyceride concentrations, we have explored the triglyceride hydrolyzing system by measuring tissue lipoprotein lipase activities and plasma activator for the enzyme. Lipoprotein lipase activities of epididymal adipose tissue of these animals were 759±117 (mean±SE) n moles FFA·min^{-1}·g wet tissue^{-1}, markedly low compared with those of rats. There were no relationships between plasma triglyceride concentrations and tissue lipase activities. Plasma activator for lipoprotein lipase was lacking in this animal. Guinea pigs with ascorbic acid deficiency for 2 weeks also showed marked variations of plasma triglyceride concentrations, without any changes in tissue lipoprotein lipase activities. Low adipose tissue lipoprotein lipase activities with deficient plasma activator for the enzyme suggest that the lipoprotein lipase-mediated triglyceride degradation could be impaired in this animal, and this may account for the marked variation of plasma triglyceride concentrations.
Lipids 19:978-981, 1984.

INTRODUCTION

We have observed previously that guinea pigs fed a high fat diet showed chylomicronemia (1). In subsequent studies, we often have observed that some of the guinea pigs fed a laboratory chow had hyperlipidemia, even if blood was taken after an overnight fast. This prompted us to perform studies focusing on plasma levels of triglyceride in this animal. Further, we have studied the triglyceride-hydrolyzing system, by measuring tissue lipoprotein lipase (LPL) activities and plasma activators for LPL.

The guinea pig is unable to synthesize ascorbic acid. Some reports suggest that chronic ascorbic acid deficiency causes changes of plasma cholesterol levels in guinea pigs (2-4), while little attention has been paid to the changes in plasma triglyceride levels. In the present work, we also have studied the effect of acute ascorbic acid deficiency on both plasma triglyceride levels and tissue LPL activities.

MATERIALS AND METHODS

Hartley strain male guinea pigs, weighing around 200 g, were used. The animals were fed an ascorbic acid-free diet containing 5% of soybean oil for 2 weeks. One group of animals received 5 mg of ascorbic acid by intubation every day. The other group of animals did not receive ascorbic acid. At the time of the experiment, the animals were fasted for 24 hr and killed by decapitation. Blood was collected into tubes containing EDTA (1 mg per ml of blood) and the plasma separated by centrifugation was used for the determination of triglyceride (5).

Six guinea pigs receiving ascorbic acid and eight ascorbic acid deficient animals were used for further studies. Both heart and epididymal adipose tissue were excised immediately and the LPL activities measured. In these animals, plasma triglyceride, cholesterol (6) and phospholipids (7) were determined enzymatically.

The whole heart was homogenized in 10 ml of 0.05 M NH_4OH-NH_4Cl buffer (pH 8.5) containing 0.5 U/ml of heparin (Novo Indust., Denmark) on ice for 60 min. The suspension was then centrifuged at 4 C, and the supernatant obtained was used for the measurement of LPL activities.

In the case of epididymal adipose tissue, an acetone-ether dried powder was prepared (8) and the LPL was then extracted from this dried powder with the same method used for the extraction of heart LPL.

The enzyme activity was determined by the method described previously (1). The substrate was a mixture of 2μCi of glycerol tri-[1-^{14}C] oleate (Amersham/Seale Corp., Arlington Heights, Illinois), 0.133 g of unlabeled triolein (Sigma Chemical Co., St. Louis, Missouri), 0.9ml of 1% Triton X-100 and 10.2 ml of 0.2 M Tris-HCl buffer (pH 8.6). The mixture was sonicated on ice for 3 min with a Tomy UP-sonifier (Tomy,

*To whom correspondence should be addressed.

Seiko Co., Tokyo). To 0.4 ml of this substrate, 0.1 ml of pooled rat plasma as an activator for lipoprotein lipase and 0.3 ml of 4% bovine serum albumin/0.2 M Tris-HCl buffer (pH 7.4) were added. Incubation was started with the addition of 0.2 ml of the enzyme extract and was carried out for 30 min at 37 C. The free fatty acids (FFA) released during incubation were extracted and the radioactivity was determined (9). The LPL activity was expressed as n moles FFA hydrolyzed per min per g wet tissue.

All results were expressed as mean±SE. The statistical significance of the data was analyzed by the Student's t test.

RESULTS

Plasma Lipid Concentrations

As Figure 1 shows, guinea pigs had wide variations of plasma triglyceride concentrations, ranging from 28 to 1392 mg/dl. Plasma cholesterol and phospholipid levels of guinea pigs were 60±9 and 66±6 mg/dl, respectively (Table 1).

LPL Activities in Epididymal Adipose Tissue and Heart

LPL activities of adipose tissue and heart were 130±13 and 759±117 n moles FFA·min^{-1}·g

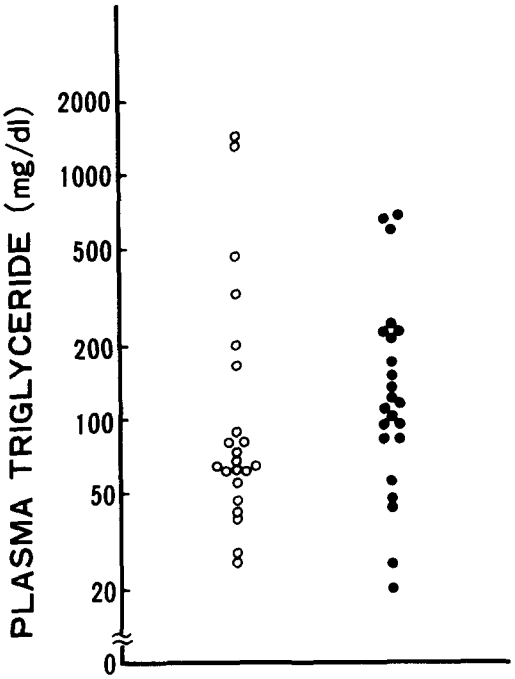

FIG. 1. Plasma triglyceride concentrations of ascorbic acid-supplemented (○) and ascorbic acid-deficient (●) guinea pigs. Values are plotted on a logarithmic scale.

TABLE 1

Plasma Lipid Concentrations and Tissue Lipoprotein Lipase Activities in Guinea Pigs

	Plasma lipids (mg/dl)			Tissue lipase activities (n moles FFA·min^{-1}·g wet tissue^{-1})	
	TG	Chol	PL	Adipose tissue	Heart
Guinea pigs					
1	46	50	48	160	858
2	1392	105	283	118	526
3	62	42	44	129	1229
4	164	52	59	84	468
5	73	49	48	172	600
6	77	59	49	115	875
Mean±SE	302±218	60±9	89±39	130±13	759±117
Ascorbic acid deficient guinea pigs					
1	82	53	58	175	868
2	96	89	79	123	799
3	242	67	99	159	711
4	589	61	143	212	599
5	232	49	83	113	492
6	119	51	64	114	536
7	171	96	122	254	584
8	96	65	73	185	774
Mean±SE	203± 72	66±6	90±10	167±18	670± 48

TG = triglyceride; Chol = cholesterol, and PL = phospholipids.

wet tissue^{-1}, respectively. Since guinea pig plasma lacks an activator for LPL, we assumed that, in compensation for this, tissue LPL activities would be increased. Therefore, for comparison purposes, we measured tissue enzyme activities of rats of similar ages. Contrary to our assumption, the adipose tissue LPL activity of guinea pigs was only one-half that of rats (262±10 n=5) and the heart LPL activity was almost comparable to that of rats (758±26).

Activation of LPL by Guinea Pig Plasma

In our previous studies using the same assay procedure, guinea pig postheparin plasma LPL achieved its full activity when 10μl of rat serum was added to the assay system (1). In the present experiment, we used LPL extracted from guinea pig heart and added to the assay system 100μl of plasma, a sufficient amount of plasma giving full LPL activity. Figure 2 shows that LPL was markedly activated by pooled rat plasma, whereas activation of LPL by pooled guinea pig plasma was very weak, and was only one-tenth of that by rat plasma.

Relationship Between Plasma Triglyceride Concentrations and Tissue LPL Activities

There were no relationships between plasma triglyceride concentrations and adipose tissue LPL activities (r=0.24) as well as heart LPL (r=0.45).

Ascorbic Acid Deficient Guinea Pigs

Ascorbic acid deficiency was evaluated in some of the guinea pigs by measuring tissue ascorbic acid contents with the use of high-performance liquid chromatography (HPLC) (10). We always have observed that ascorbic acid deficient guinea pigs gain body weight for the first 10 days and after that they begin to lose body weight rapidly (4). Ascorbic acid deficient guinea pigs showed marked variations with plasma triglyceride concentrations of between 20 and 589 mg/dl (Fig. 1). Ascorbic acid deficiency did not cause any changes in LPL activities of either adipose tissue or heart (Table 1). There were no relationships between plasma triglyceride concentrations and activities of adipose tissue LPL (r=0.32) as well as heart LPL (r=0.41) in ascorbic acid deficient animals.

DISCUSSION

The present study demonstrates that wide variations of plasma triglyceride concentrations are a characteristic feature of guinea pigs. The present study as well as previous reports (1,11,12) indicates that plasma of this animal

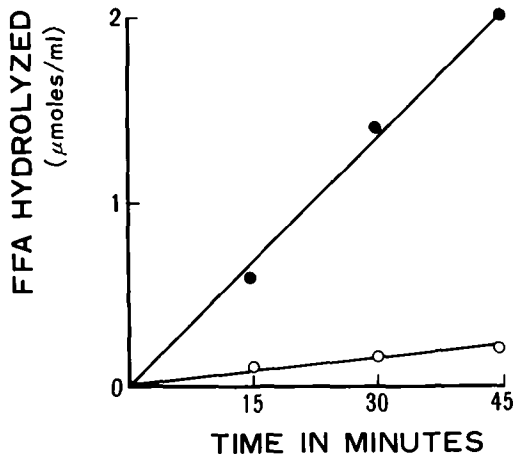

FIG. 2. Activation by guinea pig plasma (○) and rat plasma (●) of lipoprotein lipase extracted from guinea pig heart. In this experiment, 100μl of either pooled guinea pig plasma or pooled rat plasma was added to the incubation mixture.

lacks an activator(s) for LPL. We assumed that deficient plasma activator for LPL might cause a compensatory increase in tissue LPL activities. Contrary to our assumption, LPL activities in adipose tissue, which is known to have the highest LPL activity, did not increase, or rather seemed to be low. Low adipose tissue LPL activities with deficient plasma activator suggest that the LPL-mediated triglyceride removal process could be operating poorly in this animal. Because of this, if guinea pigs take increased amounts of exogenous fat or if endogenous triglyceride production is enhanced in this animal, chylomicron or very low density lipoprotein may remain for a long time in circulating plasma. Our previous observation that guinea pigs fed a high fat diet showed chylomicronemia (1) is in support of such an idea. In summary, it can be speculated that a variety of the degree of either or both exogenous fat ingestion and endogenous triglyceride production, in combination with the derangement of catabolic degradation of triglyceride due to defective LPL functions, causes wide variations of plasma triglyceride concentrations in this animal.

We also have attempted in the present study to see the effects of ascorbic acid deficiency on both plasma lipid levels and tissue LPL activities. Some authors described that chronic ascorbic acid deficiency caused an elevation of plasma cholesterol (2-4). Contradictory results have been published on the effect of acute ascorbic acid deficiency on plasma cholesterol levels (13). On the other hand, not so much attention has been paid to the effect of ascorbic acid deficiency on plasma triglyceride levels.

Fujinami et al. reported that serum triglyceride concentrations rose slightly after 2 weeks of an ascorbic acid-free diet (14). Nambisan and Kurup reported that ascorbic acid deficiency of 4 mo duration provoked hypertriglycidemia in guinea pigs (15). It is speculated that such changes may be accompanied by the changes in tissue LPL activities (13). In the present study, however, we did not observe that ascorbic acid deficiency for 2 weeks caused any changes in either plasma lipid levels or tissue LPL activities. The most striking finding is, again, that ascorbic acid-deficient guinea pigs showed varying plasma triglyceride concentrations.

In the present study, 6 of 45 guinea pigs had hyperlipidemia with plasma triglyceride concentrations of more than 400 mg/dl. Previous reports showed relatively uniform plasma triglyceride levels in guinea pigs. It is possible that in most of the previous reports hypertriglyceridemic animals were excluded as exceptional cases. However, the present study points out that the guinea pig is an unique animal in its plasma triglyceride metabolism in that the LPL-mediated triglyceride degradation should be defective and hence wide variations in plasma triglyceride concentrations are a characteristic feature of this animal.

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REFERENCES

1. Yamada, N., Murase, T., Akanuma, T., Itakura, H., and Kosaka, K. (1979) Biochim. Biophys. Acta 575, 128-134.
2. Ginter, E., Cerven, J., Nemec, R., and Mikus, L. (1971) J. Clin. Nutri. 24, 1238-1245.
3. Sulkin, N.M., and Sulkin, D.F. (1975) Ann. N.Y. Acad. Sci. 258, 317-328.
4. Holloway, D.E., Peterson, F.J., Prigge, W.F., and Gehard, R.L. (1981) Biochem. Biophys. Res. Commun. 102, 1283-1289.
5. Bucolo, G., and David, H. (1973) Clin. Chem. 19, 476-482.
6. Allain, C.C., Pron, L.S., Chan, C.S.S., Richmond, W., and Fu, P.C. (1974) Clin. Chem. 20, 470-475.
7. Takayama, M., Ito, S., Nagasawa, T., and Tanimizu, I. (1977) Clin. Chim. Acta 79, 93-98.
8. Salamon, M.R., and Robinson, D.S. (1966) Biochem. J. 99, 604-647.
9. Schotz, M.C., Garfinkel, A.S., and Huebotter, R.J. (1970) J. Lipid Res. 11, 68-69.
10. Otsuka, M., Kurata, T., Suzuki, E., Arakawa, N., and Inagaki, C. (1981) J. Nutr. Sci. Vitaminol. 27, 9-15.
11. Thomas, F.W. Jr., and Felts, J.M. (1970) Circ. Res. 26, 545-551.
12. Chapman, J., Mills, G.L., and Ledford, J.H. (1975) Biochem. J. 149, 423-436.
13. Ginter, E. (1978) Adv. Lipid Res. 16, 167-220.
14. Fujinami, T., Okada, K., Senda, K., Sugimura, K., and Kishikawa, M. (1971) Jap. Circul. J. 35, 1559-1565.
15. Nambisan, B., and Kurup, P.A. (1975) Atherosclerosis 22, 447-461.

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The Positional Distributions of Fatty Acids in the Triacylglycerols and Phosphatidylcholines of the Intestinal and Popliteal Lymph and Plasma of Sheep

W.W. CHRISTIE, R.A. CLEGG, D.T. CALVERT and R.C. NOBLE, *Hannah Research Institute, Ayr, KA6 5HL, Scotland*

ABSTRACT

As part of a study of the contribution of the intestinal lymph lipoproteins and their lipid constituents to the plasma lipids in sheep, the positional distributions of the fatty acids in the triacyl-*sn*-glycerols and phosphatidylcholines in very low density/low density lipoprotein and high-density lipoprotein fractions were determined by stereospecific analysis procedures. The triacyl-*sn*-glycerols of these lipoprotein fractions in intestinal lymph did not differ appreciably in structure and resembled the plasma triacyl-*sn*-glycerols in the composition of position *sn*-2 especially. However, there were appreciable amounts of the essential fatty acid, linoleic acid, in positions *sn*-1 and *sn*-3 of the triacylglycerols in lymph but not in plasma. This result is discussed in terms of the metabolism of the triacylglycerols of lymph after they enter the plasma as part of a mechanism for the conservation of essential fatty acids in ruminants. No differences of metabolic note were observed in the structures of the phosphatidylcholines between lipoprotein fractions and among tissues.
Lipids 19:982-986, 1984.

INTRODUCTION

In order to assess the potential contribution of the lipids and lipoproteins of the intestinal lymph to those of the plasma in sheep, the main lipoprotein fractions of the intestinal lymph, peripheral (popliteal) lymph and plasma have been isolated and their lipid class and fatty acid compositions determined (1). Triacylglycerols and phosphatidylcholines are among the most abundant glycerolipids in these body fluids. Comparative studies have shown that the structures of such lipids, i.e. the distributions of the fatty acids among the various positions of the *sn*-glycerol moieties, can vary markedly in different tissues, and that such variations can reflect important metabolic differences in aspects of the biosynthesis or catabolism of the lipids (2-4). Therefore, for comparison purposes, the positional distributions of the fatty acids in the triacyl-*sn*-glycerols and phosphatidylcholines of the main lipoprotein fractions of intestinal lymph, popliteal lymph and plasma have been determined by stereospecific analysis procedure.

EXPERIMENTAL METHODS

Lymph and Plasma Samples

Intestinal lymph, popliteal lymph and plasma were obtained from mature wethers of the Clun Forest breed as described earlier (1).

Preparative Precipitation of Lipoprotein Fractions

Chylomicrons, very low density lipoproteins (VLDL) and low density lipoproteins (LDL) were precipitated at room temperature by the addition of high moledular-weight dextran sulphate (0.05% w/v; av. mol. wt 5×10^5, Na^+ salt, Sigma London Chemical Co., Poole, Dorset) and $CaCl_2$ (0.1M), and were collected by centrifugation; the supernatant was used for isolation of the high density lipoproteins (HDL) as below (5). On redissolving the precipitated VLDL-LDL fraction in 10% (w/v) sodium sulphate solution (40ml/l of lymph or serum), the Ca^{2+} remained as its insoluble sulphate. The HDL fraction was precipitated from the VLDL-LDL supernatant by increasing the dextran sulphate and $CaCl_2$ concentrations to 0.55 (w/v) and 0.2M respectively. HDL, but not Ca^{2+}, was selectively re-dissolved from the pellet in 0.5M potassium oxalate (40ml/l of lymph or serum). Ca^{2+}-free lipoproteins then were dialyzed into 20mM Tris-HCl buffer (pH 7.2)-1% NaCl at a dilution equivalent to the original volume of serum or lymph and were reprecipitated by the addition of $CaCl_2$ as above. The precipitates were treated as above but instead of being diluted were dialyzed into 1% BaCl-1% NaCl solution to precipitate dextran sulphate and finally, after centrifugation, into 20mM Tris-HCl buffer (pH 7.7), containing 1% NaCl, 1mM EDTA and 0.02% sodium azide. Lipoproteins were stored at 4 C in this buffer and were characterized by agarose-gel electrophoresis.

Stereospecific Analysis of the Triacyl-*sn*-Glycerols

Lipids were extracted from lipoprotein frac-

tions as described earlier (1). Triacylglycerols were isolated from the total lipids in each instance by preparative thin-layer chromatography (TLC) on 0.5mm silica gel G layers (E. Merck, Darmstadt); hexane-diethyl ether (4:1, v/v) was the developing solvent. They were subjected to stereospecific analysis by a procedure described elsewhere (6). In brief, triacylglycerols were partially hydrolyzed by means of ethyl magnesium bromide, and the 1,2- and 2,3-diacyl-*sn*-glycerols formed were isolated and converted synthetically into phosphatidylcholines, which in turn were reacted with the stereospecific phospholipase A of snake venom. Results for position *sn*-1 were obtained by analysis of the lysophosphatide produced, those for position *sn*-2 were obtained independently by means of pancreatic lipase hydrolysis and those for position *sn*-3 were calculated by difference from the known triacylglycerol composition. Analyses were accepted only when they conformed to the standards of accuracy described earlier (6).

Stereospecific Analysis of the Phosphatidylcholines

Phosphatidylcholines were isolated by preparative TLC on silica gel G layers; chloroform-methanol-water (25:15:4 by vol) was the developing solvent. They were hydrolyzed by means of the phospholipase A of snake venom and the lysophospholipid (position *sn*-1) and the free fatty acids (position *sn*-2) released were isolated for analysis (7).

Gas-Liquid Chromatographic (GLC) Analysis of the Fatty Acids

Glycerolipids were transesterified with sodium methoxide (8), and free fatty acids were methylated with methanol-sulphuric acid (6). Methyl esters were subjected to GLC analysis on a column (3m x 4mm) packed with 10% Silar 10C on Gaschrom Q (Applied Science Laboratories Inc., State College, Pennsylvania, USA), maintained isothermally at 210 C with nitrogen as carrier gas.

RESULTS AND DISCUSSION

Lipoprotein fractions were isolated in this study by means of precipitation with dextran sulphate and calcium chloride, rather than by differential centrifugation, in order to obtain sufficient material (30-50mg of triacylglycerol) for structural analysis with the maximum economy of effort and the minimum use of valuable time on the ultracentrifuge. This technique gave a combined VLDL-LDL fraction, although with the plasma only the VLDL frac-

TABLE 1

Positional Distributions of Fatty Acids (mol. % of the total) in Triacyl-*sn*-Glycerols of Lipoprotein Fractions from Plasma and Intestinal Lymph

	Plasma VLDL + LDL[a]				Intestinal lymph VLDL and LDL[b]				Intestinal lymph HDL[a]			
Fatty acid	TG[c]	*sn*-1	*sn*-2	*sn*-3	TG	*sn*-1	*sn*-2	*sn*-3	TG	*sn*-1	*sn*-2	*sn*-3
14:0	2.2	1.9	2.6	2.1	1.7	1.0	2.2	1.6	1.3	0.9	2.0	1.0
15br[d]	3.6	4.1	5.3	1.5	3.7	2.8	4.2	4.1	2.6	2.2	2.1	3.4
15:0	2.7	3.7	3.9	0.6	2.7	1.5	4.5	2.2	2.3	1.8	1.6	3.7
16br	0.9	1.1	1.7	–	1.3	1.0	1.1	1.8	1.0	0.7	0.5	1.7
16:0	24.4	13.9	43.8	15.5	31.2	19.2	52.7	20.5	31.6	24.5	51.6	18.6
16:1	1.0	1.6	1.4	0.1	1.3	0.8	1.1	1.9	0.9	1.3	1.1	0.3
17br	3.4	5.1	3.5	0.7	4.2	5.7	2.0	5.0	3.4	3.2	2.4	4.5
17:0	1.4	1.9	1.4	0.8	1.4	2.1	0.8	1.3	1.1	1.7	1.1	0.4
17:1	0.9	0.4	1.1	1.2	0.7	0.5	0.5	1.0	0.9	0.8	0.6	1.2
18:0	34.4	51.6	8.8	42.7	19.8	38.1	4.3	16.9	10.6	21.2	7.6	3.0
18:1	17.6	9.9	14.4	28.4	19.9	19.3	11.3	29.1	25.3	28.2	14.1	33.6
18:2	3.3	1.4	7.0	1.6	7.2	5.0	9.1	7.6	8.5	5.6	7.5	12.3
18:3 (n-6)	0.6	0.4	0.4	0.9	0.5	0.5	0.1	0.6	0.2	0.3	0.2	0.2
18:3 (n-3)	0.7	0.4	1.4	0.4	2.5	1.7	2.2	3.6	3.6	2.3	1.6	7.0
20:3 (n-6)	0.5	0.2	0.2	0.9	0.3	0.3	0.3	0.2	0.3	0.2	0.2	0.5
20:4 (n-6)	1.1	1.1	1.2	0.9	1.0	0.3	1.3	1.3	0.8	0.9	0.8	0.7
20:5 (n-3)	0.8	–	0.9	1.4	0.3	0.2	0.3	0.4	1.2	0.8	0.5	2.2
22:5 (n-3)	0.4	–	0.7	0.3	0.4	0.1	0.8	0.3	2.0	0.9	1.3	3.8
22:6 (n-3)	0.2	–	0.5	0.2	0.4	0.1	1.0	0.2	2.6	2.5	3.2	2.0

[a]Pooled sample from 5 sheep.

[b]Mean of analyses of 5 separate samples.

[c]TG = triacylglycerols.

[d]br = branched-chain fatty acid.

TABLE 2

Positional Distributions of Fatty Acids (mol. % of the total) in Phosphatidylcholines of Lipoprotein Fractions from Plasma, and Intestinal and Popliteal Lymph (Analyses of pooled samples from 5 sheep were carried out in duplicate).

	Plasma VLDL and LDL			Plasma HDL			Intestinal lymph[a] VLDL and LDL			Intestinal lymph HDL			Popliteal lymph VLDL and LDL			Popliteal lymph HDL		
Fatty acid	PC[b]	*sn*-1	*sn*-2	PC	*sn*-1	*sn*-2	PC	*sn*-1	*sn*-2	PC	*sn*-1	*sn*-2	PC	*sn*-1	*sn*-2	PC	*sn*-1	*sn*-2
14:0	0.6	0.5	0.4	0.3	0.4	0.1	0.9	0.8	0.5	1.2	0.6	0.4	0.7	1.8	0.5	0.5	1.1	0.2
15:0	0.7	1.6	0.6	0.6	1.4	0.2	1.2	1.5	0.5	0.9	1.7	0.3	0.2	1.3	0.6	0.2	1.0	0.1
16:0	18.7	32.7	4.9	16.4	30.3	2.6	21.7	37.7	6.7	26.6	43.6	5.7	24.0	36.8	8.8	22.2	39.0	2.3
16:1	1.0	1.5	1.4	1.7	1.7	0.6	0.8	0.6	0.7	1.0	1.3	1.0	1.5	0.8	2.0	0.8	0.8	1.0
17br[c]	0.7	1.8	0.1	0.8	2.5	0.5	1.5	2.8	0.9	1.8	2.6	0.1	1.3	3.0	–	1.6	3.4	0.2
17:0	1.0	2.4	0.6	1.2	2.2	0.2	1.0	2.0	0.5	1.1	1.9	0.1	0.6	1.6	0.6	0.7	1.4	0.7
18:0	23.0	50.4	3.1	24.9	49.4	3.0	17.8	40.0	2.6	13.9	25.3	2.1	20.9	46.0	1.9	20.3	43.0	0.4
18:1	18.7	4.7	27.0	18.3	5.8	28.1	16.7	9.4	19.6	16.9	16.8	16.0	20.5	6.5	31.8	20.5	7.7	31.7
18:2	23.4	3.1	44.8	19.5	1.1	37.0	25.2	3.4	46.2	24.5	3.1	51.0	16.4	0.9	29.8	15.7	1.4	31.9
18:3	2.2	0.2	3.4	2.5	0.3	4.8	3.0	0.7	5.0	2.8	0.9	5.0	1.3	0.2	1.4	1.2	0.5	1.5
20:3 (n-6)	0.5	0.1	0.4	0.8	0.3	0.9	0.3	–	0.5	0.3	0.1	0.4	0.8	–	1.2	1.1	–	2.0
20:3 (n-3)	0.5	0.1	0.9	0.4	0.3	1.0	0.4	–	0.7	0.4	0.4	0.7	1.0	0.1	1.7	1.2	–	2.4
20:4 (n-6)	4.0	0.3	6.5	6.5	1.8	10.8	4.9	0.6	8.2	4.1	0.5	8.7	5.4	0.2	8.7	8.3	0.3	14.1
20:5 (n-3)	1.0	0.3	1.0	1.2	–	2.1	1.3	0.1	2.1	1.5	0.1	2.9	0.8	–	3.6	1.0	0.1	1.7
20:5 (n-3)	1.5	–	2.2	1.4	0.9	2.8	1.5	0.2	2.3	1.4	0.2	2.6	2.1	0.2	3.0	1.8	–	4.0
22:6 (n-3)	1.3	0.1	1.7	2.6	1.6	2.5	1.6	0.4	2.5	1.4	0.7	2.9	1.5	0.1	2.3	1.8	–	3.3

[a]Mean of analyses of samples from 5 individual sheep.

[b]PC = phosphatidylcholine.

[c]br = branched-chain fatty acid.

tion contained any triacylglycerol (1). It was still necessary to pool material from several animals in some instances to obtain sufficient lipid for analysis. Unfortunately, some minor differences in fatty acid compositions between corresponding lipids isolated by the two procedures were observed, and these must be considered when the results are interpreted.

The positional distributions of the fatty acids in the triacyl-*sn*-glycerols in the VLDL-LDL fraction of plasma, and the VLDL-LDL and the HDL fractions of intestinal lymph are listed in Table 1; insufficient triacylglycerol for analysis was present in popliteal lymph. In each instance, palmitic acid and the other C_{14} to C_{17} saturated components were in greatest abundance in position *sn*-2, while stearic and oleic acids were concentrated in the primary positions, the former predominantly in position *sn*-1 and the latter predominantly in position *sn*-3. Although there are some minor differences in these components among triacylglycerols from the 3 sources, it does not appear likely that they reflect important metabolic differences.

There was much less linoleic acid in the triacylglycerols of plasma than in those of intestinal lymph, and this also was found when the fractions were isolated by differential centrifugation (1). However, similar concentrations of linoleic acid were found in position *sn*-2 in each instance. In contrast, very little of this component was present in the primary positions of the plasma triacylglycerols although appreciable amounts were found in both positions *sn*-1 and *sn*-3 of the triacylglycerols of the intestinal lymph lipoproteins.

The positional distributions of the fatty acids in the phosphatidylcholines of the VLDL-LDL and HDL fractions of plasma, intestinal lymph and popliteal lymph are listed in Table 2. Although there were some minor differences in overall fatty acid compositions, the relative distributions of fatty acids between positions *sn*-1 and *sn*-2 did not differ appreciably between samples. In each instance, the saturated fatty acids tended to be concentrated in position *sn*-1 and the unsaturated in position *sn*-2.

A similarity in the positional distribution of fatty acids in the triacyl-*sn*-glycerols of sheep lymph and plasma has been noticed before, and this was taken as confirmation of their common origin (9-11). In particular it was noted that palmitic acid was concentrated in position *sn*-2, a feature not found in other triacylglycerols of sheep tissues. It was not apparent from the earlier analyses, either because samples were from different groups of animals or because full stereospecific analyses were not carried out, that there were major differences in the compositions of the primary positions. In particular, relatively high proportions of the essential fatty acid, linoleic acid, were present in positions *sn*-1 and *sn*-3 of the lymph triacylglycerols but not in the plasma triacylglycerols. The most plausible explanation would appear to be that substantial hydrolysis of the fatty acids from the primary positions of the lymph triacylglycerols occurs after they enter the plasma, followed by resynthesis of triacylglycerols from the intermediate 2-monoacyl-*sn*-glycerols and the more saturated fatty acid components. The essential fatty acids might then be retained for the synthesis of membrane components and related functions. As a consequence of biohydrogenation in the rumen, the essential fatty acids are in relatively short supply in the tissues of ruminants, where they appear to be directed away from nonessential functions (12,13). Lipoprotein lipase in the peripheral tissues reacts most rapidly with the fatty acids in the primary positions, generating 2-monoacyl-*sn*-glycerols that potentially could be used in the manner above (the properties of the enzyme have been reviewed) (14-15). That 2-monoacylglycerols are not found in ruminant plasma is perhaps not surprising in view of the very low levels of triacylglycerols present. Hepatic lipase also could perform this hydrolytic function, and its presence in ruminant liver recently has been confirmed (16), in contradiction of an earlier negative report (17). The liver would certainly appear to be the most likely site for the putative reesterification of the monoacylglycerols to triacylglycerols. Triacylglycerols of this kind with the essential fatty acids located predominantly in position *sn*-2 would not be able to supply these components as readily to milk fat or adipose tissue, where concentrations of only 1-2% of linoleic acid in the total fatty acids generally are encountered. The phenomenon observed here may then be part of the mechanism for conserving essential fatty acids in ruminants.

There appeared to be no difference of metabolic significance between the structures of the triacylglycerols of different lipoprotein fractions in the intestinal lymph, or in the structures of the phosphatidylcholines in different lipoprotein fractions in plasma, popliteal lymph and intestinal lymph.

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REFERENCES

1. Noble, R.C., Shand, J.H., Calvert, D.T., Clegg,

R.A., and Christie, W.W. (1984) J. Sci. Fd. Agric. 35, 1083-1091.
2. Christie, W.W. (1981) in Lipid Metabolism in Ruminant Animals (Christie, W.W., ed.) p. 95-191, Pergamon Press, Oxford.
3. Holub, B.J., and Kuksis, A. (1978) Adv. Lipid Res. 16, 1-125.
4. Breckenridge, W.C. (1978) in Handbook of Lipid Research (Kuksis, A., Ed.) Vol. 1, Fatty Acids and Glycerides, p. 197-232, Plenum Press, New York, NY.
5. Burstein, M., and Scholnick, H.R. (1973) Adv. Lipid Res. 11, 67-108.
6. Christie, W.W. (1982) Lipid Analysis, Pergamon Press, Oxford.
7. Robertson, A.F., and Lands, W.E.M. (1962) Biochemistry 1, 804-810.
8. Christie, W.W. (1982) J. Lipid Res. 23, 1072-1075.
9. Garton, G.A., and Duncan, W.R.H. (1965) Biochim. Biophys. Acta 98, 436-438.
10. Christie, W.W., and Moore, J.H. (1971) J. Sci. Fd. Agric. 22, 120-124.
11. Christie, W.W., and Hunter, M.L. (1978) J. Sci. Fd. Agric. 29, 442-446.
12. Christie, W.W. (1981) in Lipid Metabolism in Ruminant Animals (Christie, W.W., ed.) p. 193-226, Pergamon Press, Oxford.
13. Noble, R.C. (1984) in Fats in Animal Nutrition (Proc. 37th Easter School in Agricultural Science, University of Nottingham) (Wiseman, J., ed.), Buttersworth, London, p. 185-200.
14. Nilsson-Ehle, P., Garfinkel, A.S., and Schotz, M.C. (1980) Ann. Rev. Biochem. 49, 667-693.
15. Cryer, A. (1981) Int. J. Biochem. 13, 525-541.
16. Cordle, S.R., Yeaman, S.J., and Clegg, R.A. (1983) Biochim. Biophys. Acta 753, 213-219.
17. Etienne, J., Noe, L., Rossignol, M., Dosne, A.-M., and Debray, J. (1981) Biochim. Biophys. Acta 663, 516-523.

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Activation of Polyunsaturated Fatty Acids by Rat Tissues in vitro

T. SANJEEVA REDDY and **NICOLAS G. BAZAN,*** *LSU Eye Center, Louisiana State University Medical Center School of Medicine, New Orleans, LA*

ABSTRACT

The conversion of labeled palmitic, linoleic, arachidonic and docosahexaenoic acids to their respective acyl CoA's was studied in homogenates and microsomes of rat tissues. The highest activity, both in homogenates and microsomes, was seen in liver and heart. There was moderate activity in retina, brain, lung, kidney and testes and the lowest activity was found in spleen. Docosahexaenoic acid was activated much less actively in heart tissue than the other fatty acids. In all tissues examined, the highest activation was observed with arachidonic acid and the lowest with docosahexaenoic acid. Except for liver, those tissues that contained high levels of docosahexaenoic acid also had the highest activation capacity for this fatty acid.
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INTRODUCTION

Although phospholipids of neuronal plasma membranes (1-4) and photoreceptor cell membranes (5-10) are highly enriched in polyunsaturated fatty acids, such as arachidonic acid (20:4, n-6) and docosahexaenoic acid (22:6, n-3), our understanding of the activation-acylation reactions of these fatty acids is limited. Long-chain acyl-CoA synthetase has been described in various mammalian tissues using saturated, mono- and dienoic long-chain fatty acids as substrates (11-16), but to the best of our knowledge there have been no studies on the activation of polyenoic fatty acids. In this report we describe the first step in the sequential enzymatic pathways that are involved in the incorporation of arachidonic and docosahexaenoic acids into phospholipids, by the activation reactions leading to the formation of thiol esters of Coenzyme A, in various rat tissues. Studies also were carried out on the activation of palmitic and linoleic acids for comparison.

MATERIALS AND METHODS

All *cis* (U-^{14}C) 4,7,10,13,16,19-docosahexaenoic acid (specific activity of 160 mCi/mmol), *cis* (1-^{14}C) 4,7,10,14-arachidonic acid (58 mci/mmol), *cis* (1-^{14}C) 9,12-linoleic acid (56 mci/mmol) and (1-^{14}C)-palmitic acid (59 mci/mmol) were purchased from New England Nuclear, Boston, Massachusetts. Unlabeled fatty acids were purchased from Serdary Research Laboratories, London, Ontario, Canada and Sigma Chemical Co., St. Louis, Missouri.

Male albino Wistar rats weighing 250-300 g were decapitated and the organs to be examined were removed rapidly, washed in ice-cold 0.9% saline, and homogenized immediately in 0.25 M sucrose in 10 mM Tris HCl buffer (pH 7.4) using a Potter-Elvejhem homogenizer with a motor-driven Teflon pestle. Part of the homogenate was stored at −80 C for further analysis, and the remaining quantity was centrifuged at 20,000 g for 20 min. The supernatant was centrifuged at 105,000 g for 60 min to obtain the microsomal pellet. These steps were carried out at 4 C. Microsomal fractions were suspended in a small volume of 10 mM Tris HCl buffer, pH 8.0, and stored at −80 C.

The assay was performed within 3 or 4 days after the collection of samples. Standard assay conditions described previously for brain microsomal long-chain acyl-CoA synthetase were adapted for the present studies (17). The assay mixture contained 30 μmol Tris HCl (pH 8.0), 1 μmol ATP, 500 nmol CoA, 500 nmol DDT, 4 μmol $MgCl_2$, 20 nmol radiolabeled fatty acid and enzyme protein of 200-220 μg homogenate protein or 60-65 μg microsomal protein in a total volume of 0.20 ml. All the labeled fatty acids were diluted with unlabeled fatty acids so that the radioactivity of each was between 20,000-30,000 cpm/nmol. The reaction was started by adding the enzyme protein to the previously incubated assay system (37 C for 2 min) and was allowed to continue for 1 min. The reaction was terminated by the addition of 2.25 ml of isopropanol:heptane:2 M sulfuric acid (40/10/1, v/v/v). Heptane (1.5 ml) and water (1.0 ml) were added and the mixture was vortexed vigorously. The upper phase was discarded and the lower phase (aqueous phase) was washed twice with 2 ml heptane containing 5 mg/ml carrier palmitic acid to remove the unreacted labeled fatty acid. The aqueous phase was transferred to a scintillation vial and counted in a Beckman LS-7500

*To whom correspondence should be addressed at LSU Eye Center, 136 South Roman St., New Orleans, LA 70112.

after the addition of 10 ml Ready-Solv EP scintillation fluid (Beckman Instruments, Fullerton, California). Blanks were treated in a similar manner except boiled enzyme protein was used instead of the normal enzyme protein. Protein content of the homogenate and microsomes was estimated by the method of Lowry (18).

RESULTS AND DISCUSSION

The activity of long chain acyl CoA synthetase from homogenates and microsomal fractions of rat tissues is given (Table 1). Although there have been reports on the activation of palmitic and stearic acids in rat tissues (11,14), this is the first report on the activation of polyunsaturated fatty acids. The available data on long chain acyl Coenzyme A synthetase from various tissues such as liver (13,15,19,20), brain (17,21,22), retina (23), aorta (24), lung (25) and platelets (16,22) were obtained using similar assay conditions. Therefore, the present study utilized similar optimal assay conditions for the assay of long chain acyl Coenzyme A synthetase and should reflect the optimal activities in different tissues. Among the tissues studied, the highest specific activity of long-chain acyl-CoA synthetase in homogenates and microsomes was in liver and heart and moderate activity was observed in retina, brain, lungs, kidneys and testes, with spleen showing the least activity. There was a similar pattern of tissue activity for all fatty acids studied, except for the very low activation of docosahexaenoic acid in heart tissue.

The activity of long chain acyl Coenzyme A synthetase in homogenates and microsomes of liver, brain, retina, lung, kidney and spleen with various fatty acids showed the following pattern: arachidonic ≥ linoleic acid > palmitic acid > docosahexaenoic acid. This pattern of activity is significantly different (p value less than 0.05) between different fatty acids. However, heart and testes homogenates and microsomes showed a slightly different pattern: arachidonic acid > palmitic acid > linoleic acid > docosahexaenoic acid. The pattern of activity with palmitic, linoleic and arachidonic acids in homogenates and microsomes was similar. The activity in homogenates showed the following pattern: liver > heart > kidney > brain = retina = testes > lung > spleen. The pattern for microsomal activity was: liver > heart > retina > lung > brain = kidney > testes > spleen. All these activities are significantly different (p values less than 0.05) between 2 different tissues. The activation pattern of docosahexaenoic acid was slightly different from other fatty acids. Thus, in homogenates the pattern was: liver > kidney > brain > testes > retina = heart = lung > spleen and for microsomes: liver > kidney = brain = retina = testes > heart > spleen. The difference in the pattern between 2 tissues was significantly different (p values less than 0.05).

The specific activities obtained for the tissue homogenates in the present studies are very similar to those reported previously for palmitate activation (11,14). Moreover, this is the first time that the activation of long chain fatty acids in retina and spleen have been shown. Higher activities for the activation of linoleic acid in liver and lung microsomes (13,25) and linoleic and arachidonic acids in brain and liver homogenates (26) as compared to palmitate have been reported. Slightly lower values for liver and lung microsomal arachidonate activation as compared to palmitate also have been documented (25), which is in con-

TABLE 1

Fatty Acid Activation by Rat Tissues

Fatty acid	16:0		18:2		20:4		22:6	
	H	M	H	M	H	M	H	M
Liver	52 ± 3	121 ± 2	59 ± 2	132 ± 3	78 ± 1	136 ± 2	17 ± 0.2	43 ± 1
Heart	14 ± 0.5	64 ± 0.5	14 ± 1	58 ± 1	24 ± 0.8	70 ± 1	1.3 ± 0.1	5.5 ± 0.2
Retina	3.3 ± 0.2	18 ± 0.9	5.3 ± 0.2	22 ± 1	7.8 ± 1	21 ± 0.6	1.5 ± 0.2	7.5 ± 1.5
Brain	4.3 ± 0.1	10 ± 0.1	5.3 ± 0.1	12 ± 0.1	7.7 ± 0.1	16 ± 0.6	3.4 ± 0.1	8.1 ± 0.3
Lung	1.9 ± 0.1	16 ± 0.8	2.8 ± 0.3	16 ± 1	5.6 ± 0.3	19 ± 0.7	1.0 ± 0.2	6.8 ± 0.3
Kidney	5.6 ± 0.1	9.9 ± 0.2	7.3 ± 0.2	11 ± 0.2	10 ± 0.4	18 ± 0.5	4.9 ± 0.2	9.3 ± 0.5
Testes	4.7 ± 0.3	8.2 ± 0.2	3.7 ± 0.1	6.5 ± 0.3	7.1 ± 0.2	16 ± 0.3	2.5 ± 0.2	6.6 ± 0.2
Spleen	1.2 ± 0.1	3.0 ± 0.1	1.8 ± 0.1	3.9 ± 0.6	3.3 ± 0.1	4.7 ± 0.6	0.7 ± 0.1	1.8 ± 0.2

Values expressed as nmol/min/mg protein and are mean ± S.E. of 4 separate incubations. The details of the assay system are given in text.

H, homogenate, M; microsomes, 16:0, palmitic acid; 18:2, linoleic acid; 20:4, arachidonic acid; 22:6, docosahexaenoic acid.

trast to our results. However, a previous report on much lower values for docosahexaenoic acid activation in liver microsomes as compared to other long chain fatty acids is in good agreement with our results (13).

There was no correlation between the distribution of polyunsaturated fatty acids in different tissues and their rate of activation by long-chain acyl-CoA synthetase. Thus, liver lipids are not enriched in polyunsaturated fatty acids, despite the high activation capacity of this tissue for arachidonic and docosahexaenoic acids. This high activation capacity may be responsible, in part, for the retention of these fatty acids for further distribution via the blood stream to other organs. However, it is interesting that organs such as retina (5,10), brain (1-4) and testes (27) with high amounts of docosahexaenoic acid esterified to complex lipids showed the highest activity, with the exception of liver, for the docosahexaenoic acid activation reaction. Also, the heart, which utilizes palmitic, linoleic and arachidonic acids more efficiently than all other tissues examined, except liver, seems to be a poor site for the activation of docosahexaenoic acid.

The very rapid activation of arachidonic acid in the tissue homogenates and microsomes may be a mechanism regulating the availability of this fatty acid in its free form for the synthesis of biologically important compounds, such as the eicosanoids and leukotrienes (17,26).

In conclusion, activation of polyunsaturated fatty acids was observed in all rat tissues studied. The pattern of activation appears to be similar to that reported for saturated fatty acids, except for the activation of docosahexaenoic acid in heart tissue, which is very low compared with other fatty acids. With the exception of liver, the tissues that were rich in docosahexaenoic acid had a high capacity for its activation reaction. Even though the activity of long chain acyl Coenzyme A synthetase with docosahexaenoic acid is low, its affinity towards 22:6 is higher (lower Km) compared to other fatty acids such as arachidonic acid and palmitic acid in brain (17,21) and retina (23).

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REFERENCES

1. Breckenridge, W.C., Gombos, G., and Morgan, I.G. (1971) Brain Res. 33, 581-586.
2. Cotman, C., Blank, M.L., Moehl, A., and Snyder, F. (1969) Biochemistry 8, 4606-4612.
3. Kishimoto, Y.L., Agranoff, B.W., Radin, N.S., and Burton, R.M. (1969) J. Neurochem. 16, 397-405.
4. Sun, G.Y., and Sun, A.Y. (1972) Biochim. Biophys. Acta 280, 306-315.
5. Anderson, R.E., and Maude, M.B. (1970) Biochemistry 9, 3624-3638.
6. Bazan, N.G., De Escalante, M.S.D., Careaga, M.M., Bazan, H.E.P., and Guisto, N.M. (1982) Biochim. Biophys. Acta 712, 702-706.
7. Bazan, N.G. (1982) in Phospholipids in the Nervous System, Metabolism (Horrocks, L.A., Ansell, G.B., and Porcellati, G., eds.), Vol. 1, pp. 49-62, Raven Press, New York.
8. Giusto, N.M., and Bazan, N.G. (1979) Biochem. Biophys. Res. Commun. 91, 791-794.
9. Stone, W.L., Farnswoth, C.C., and Dratz, E.A. (1979) Exp. Eye Res. 28, 387-397.
10. Aveldano, M.I., and Bazan, N.G. (1983) J. Lipid Res. 24, 620-627.
11. Aas, M. (1971) Biochim. Biophys. Acta 231, 32-47.
12. Groot, P.H.E., Scholte, H.R., and Hulsmann, W.C. (1976) Adv. Lipid Res. 14, 75-126.
13. Normann, P.T., Thomassen, M.S., Christiansen, E.N., and Flatmark, T. (1981) Biochim. Biophys. Acta 664, 416-427.
14. Pande, S.V., and Mead, J.F. (1968) Biochim. Biophys. Acta 152, 636-638.
15. Tanaka, A., Hosaka, K., Hoshimaru, M., and Numa, S. (1979) Eur. J. Biochem. 98, 165-172.
16. Wilson, D.B., Prescott, S.M., and Majerus, P.W. (1982) J. Biol. Chem. 257, 3510-3515.
17. Reddy, T.S., and Bazan, N.G. (1983) Arch. Biochem. Biophys. 226, 125-133.
18. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randell, R.J. (1951) J. Biol. Chem. 193, 265-275.
19. Bar-tana, J., and Rose, G. (1973) Biochem. J. 131, 443-449.
20. Irtani, N., Ikeda, Y., and Kajitani, H. (1984) Biochim. Biophys. Acta 793, 416-422.
21. Reddy, T.S., Sprecher, H., and Bazan, N.G. (1984) Eur. J. Biochem. (In press).
22. Neufeld, E.J., Sprecher, H., Evans, R.W., and Majerus, P.W. (1984) J. Lipid Res. 25, 288-292.
23. Reddy, T.S., and Bazan, N.G. (1984) Curr. Eye Res. 3, 1225-1232.
24. Morisaki, N., Saito, Y., and Kumagai, A. (1983) Biochim. Biophys. Acta 752, 301-306.
25. Okuyama, H., Yamada, K., Miyagawa, T., Suzuki, M., Prasad, R., and Lands, W.E.M. (1983) Arch. Biochem. Biophys. 221, 99-107.
26. Reddy, T.S., and Bazan, N.G. (1984) Int. J. Develop. Neurosci. 2, 447-450.
27. Grogan, W.M. (1981) Lipids 16, 940-942.

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The Occurrence of Long Chain α,ω-Diols in the Lipids of Steer and Human Meibomian Glands

N. NICOLAIDES,*[,a] EVELYN C. SANTOS,[a] KONSTANTINE PAPADAKIS,[a] EDWARD C. RUTH[b] and LUCIANO MÜLLER,[c] [a]*Department of Medicine (Dermatology), University of Southern California, School of Medicine, Los Angeles, CA 90033,* [b]*Institute of Geophysics and Planetary Physics, University of California at Los Angeles, Los Angeles, CA 90024, and* [c]*Smith, Kline and French Laboratories, Philadelphia, PA 19101*

ABSTRACT

A group of long chain α,ω-diols (C_{29} to C_{34}) has been identified in the lipids of steer and human meibomian gland excreta (meibum). These new lipids were isolated from the steer meibum unsaponifiables. Proof of structure was provided by 1) the column chromatographic behavior and TLC of the diols and their diacetates; 2) GLC on glass capillary columns; 3) fragmentation patterns in GC-MS; 4) NMR data, and 5) ozonolysis studies of the unsaturates. Chain types for the steer sample were 51% straight monoenes, 8.5% straight saturates, 39% iso and anteiso saturates and 1.5% iso and anteiso unsaturates. GC for the human sample gave straight monoenes 83%, straight saturates 8%, and iso plus anteiso saturates 9%. Close correspondence of the α,ω-diol chain lengths and types with meibum ω-hydroxy fatty acids suggests a biochemical precursor relationship.
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INTRODUCTION

Meibum, the excreta of the meibomian gland, is a unique collection of lipids, consisting primarily of sterol esters (~30%) and wax esters (~35%), with minor amounts of triacylglycerols (~2%), unesterified fatty acids (~5%) and free cholesterol (~3%). About 25% are unidentified lipids (1).

We wish to report the presence in meibum of a new lipid component; namely, a family of α,ω-diols of exceedingly long chain lengths (C_{29} to C_{34}). These lipid components are combined in some manner, to form part of the unidentified lipids of meibum. α,ω-Diols of shorter chain lengths occur in an esterified form in plants as components of the protective lipid polymer cutin (2,3). This is the first report of their occurrence in animal tissue.

MATERIALS AND METHODS

Steer meibomian gland lipids were collected (263 mg), saponified and the unsaponifiables separated from the fatty acids according to procedures already reported (1,4). The unsaponifiables (122 mg) were further separated by silicic acid column chromatography (column dimensions 20.0 cm x 2.8 cm) into fractions of fatty alcohols, sterols and α,ω-diols using hexane, followed by increasing concentrations of benzene in hexane, then by increasing concentrations of methanol in chloroform. The diols were purified further by preparative TLC, then acetylated, hydrogenated and analyzed by GC-MS, also by procedures previously reported (1,5) and by new capillary GC -MS, also by procedures previously reported (1,5) and by new capillary GC procedures (6). The latter were performed on a Varian 3700 instrument with a flame ionization detector. Injection was by means of an on-column injector (J & W Scientific, Inc., Rancho Cordova, California) using a fused silica capillary column, 25 m x 0.25 mm, with chemically bonded SE-30 phase, film thickness 0.4μ (Chromapon Inc., Whittier, California). Temperature programming was from 75 C to 320 C at 7°/min, with He the carrier gas at a flow rate of 1.8 ml/min.

The ^{1}H-NMR spectra were obtained with a JEOL GX 500 instrument operating at 500 MHz with the sample (100 μg) dissolved in $CDCl_3$ using tetramethylsilane (TMS) as the chemical shift reference.

Reductive ozonolysis was carried out by ozonizing 50 μg of the total sample of diols as the diacetates (4,7), the reductant being triphenyl phosphine.

An aliquot of a pooled sample of human meibum, obtained as previously reported (1), was subjected to TLC on three 19-channel Whatman LKD plates. Nine spots (200 μg each) of human meibum were spotted on one side of each plate while 8 spots (also 200 μg each) of steer meibum were spotted on the other side of the same plates, 2 spots being reserved for standards. Both the human and the steer samples showed 4 faint spots in the same diester region when the plate was developed with

*To whom correspondence should be addressed.

hexane/benzene (6:4, v/v), then sprayed with aniline naphthalene sulfonic acid (ANS) and viewed under UV light. Material migrating to R_f = .24 was scraped off the plate and extracted from the adsorbent with chloroform. The saponified products from both samples yielded α,ω-diols by TLC (developed with chloroform) which, when scraped off the plate, extracted and acetylated, gave the same peaks by capillary GC as those derived from the unsaponifiables of the total meibum of the steer.

RESULTS AND DISCUSSION

The following observations support the conclusion that the compounds we isolated from steer meibum were indeed a family of α,ω-diols of exceedingly long chain lengths:

The compounds were isolated from the unsaponifiable fraction of the lipid, where long chain diols and other fatty alcohols are found.

The chromatographic behavior of the α,ω-diols on silicic acid was as expected, i.e., when chromatographed on thin layer plates, the material showed a polarity greater than that of fatty alcohols with only one hydroxyl group, but slightly less than that of 1,16-hexadecane diol. Exceedingly long chain compounds generally show polarities less than those with shorter chains. Similarly, the diacetates of the isolated material with chain lengths in the range of C_{32} showed a polarity slightly less than that of 1,16-diacetoxy hexadecane.

The isolated material as the acetylated derivative gave, on analysis by GC-MS, a group of substances whose fragmentation patterns were similar to that of 1,16-diacetoxy hexadecane. The latter spectrum gave a base peak at 55 amu, a faint molecular ion, at 342 amu, loss of one or two molecules of acetic acid at M-60 or M-120 (m/z = 282 or 222 amu respectively), and loss of $O{=}CCH_3$ at M-43 (m/z = 299 amu). A typical mass spectrum for the acetylated derivative of one of the isolated substances, namely the $C_{30:1}$ alkene diol, is shown in Figure 1. Both saturates and monoenes were present, the unsaturated molecules showing 2 amu less than the corresponding saturate for M, M-60, M-120, etc. The middle part of the mass spectra of all the homologs showed a typical hydrocarbon breakdown with no unusual features such as α-cleavages, suggesting that the OH groups were not in the interior of the hydrocarbon chain.

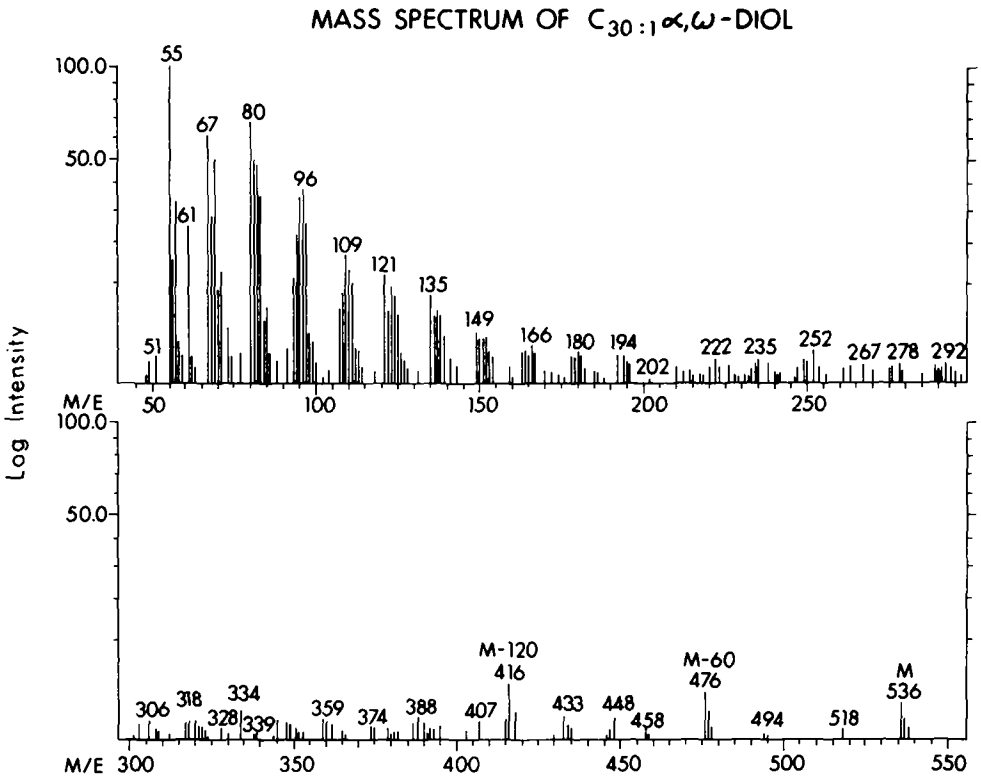

FIG. 1. Mass spectrum of $C_{30:1}$ α,ω-diol taken by GC-MS as indicated in text.

GC-MS also established the presence of 2 OH groups in each homolog and has restricted their location to either the α,ω or to the more commonly occurring α,β positions. NMR data can differentiate between these possibilities.

^{1}H NMR ($CDCl_3$) of the diols from the total unsaponifiables gave δ5.34 ppm (unresolved multiplet, 2H, olefinic), δ3.64 ppm (t,4H, HO-C$\underline{H}_2$-CH_2-), δ2.0 ppm (unresolved multiplet, 4H, HO-CH_2-C$\underline{H}_2$-), δ1.5 ppm (H_2O), δ1.26 ppm (aliphatic protons), δ0.87 ppm (d plus unresolved peaks, 3H total -CH-C$\underline{H}_3$).

This is the spectrum of an α,ω-diol since there are 4 protons in the α and β position relative to the OH group and the resolved part of the methyl signal is a doublet indicating attachment to a methine group (a terminal methyl group would display a triplet). The olefinic group must be at some distance from the chain ends because the 2 olefinic protons are strongly coupled to each other. Evaluation of peak intensities and the multiplet pattern of the CH_2-O and the methyl protons suggest the following distribution of diols; 51% of unbranched α,ω-diols, 29% of α,ω-diols with a methyl group directly attached to the otherwise unbranched hydrocarbon chain and 20% with a methyl group at the β position relative to the OH group. Peak integrals provided an approximate value of protons suggesting chain lengths of C_{28} to C_{38}. This interpretation is entirely consistent with the GC data where some of the homologs are unsaturated and unbranched while others are mainly saturated and of the iso and anteiso structure, ranging from C_{29} to C_{38}.

We also can differentiate α,ω-diols from α,β-diols of unsaturated compounds by ozonolysis studies if the OH groups are not attached to the unsaturated C-atoms. For example, reductive ozonolysis of the α,ω-diol diacetate will yield 2 different molecules of aldehyde acetate, whereas an unsaturated α,β-diol diacetate would

TABLE 1

Comparison of Composition of Long Chain α,ω-Diols and ω-Hydroxy Fatty Acids of Steer Meibum

	Straight chain			
	Saturates		Monoenes	
Chain length	α,ω-alkane diols[a] %	ω-hydroxy fatty acids[b,c] %	α,ω-alkene diols[a] %	ω-hydroxy fatty acids[b,c] %
C_{29}	0.9	tr	–	–
C_{30}	3.9	0.5	11.6	6.2
C_{31}	1.3	tr	9.2	6.1
C_{32}	2.3	0.4	24.9	39.1
C_{33}	0.1	tr	2.4	7.7
C_{34}	–	tr	3.2	23.6
C_{35}	–	tr	–	0.3
C_{36}	–	tr	–	1.6
C_{38}	–	–	–	tr
	8.5%	0.9%	51.3%	84.6%
	Branched chain			
	Saturates		Monoenes	
aiC_{29}[d]	7.3	1.0	–	–
iC_{30}	5.1	0.7	0.7	–
aiC_{31}	20.6	5.8	tr	0.1
iC_{32}	2.1	1.2	0.7	0.3
aiC_{33}	3.4	4.8	tr	0.4
iC_{34}	0.2	tr	0.1	0.2
aiC_{35}	–	tr	–	–
	38.7%	13.5%	1.5%	1.0%

[a] As diacetates.

[b] As the acetate derivative of the fatty acid methyl esters.

[c] Data from ref. 4.

[d] Assignments of iso and anteiso structures are by GC retention data only.

TABLE 2

α,ω-Diols Occurring in a Diester of Human Meibum[a]

Chain length	Straight: Saturates %	Straight: Monoenes %
C_{29}	–	–
C_{30}	6.9	19.6
C_{31}	tr	2.8
C_{32}	1.4	53.2
C_{33}	–	.9
C_{34}	–	6.6
	8.3	83.1
	Branched (iso plus anteiso)	
aiC_{29}	1.6	
iC_{30}	3.7	
aiC_{31}	2.1	
iC_{32}	1.2	
aiC_{33}	tr	
	8.6	

[a]Prepared and run as described in Materials and Methods.

yield an aldehyde plus an aldehyde with 2 acetoxy groups α,β to each other on the terminal end. When we ozonized the total acetylated sample of the isolated alkene diols we found peaks corresponding to C_7, C_8 and C_9 aldehyde acetates in the low molecular weight region, and peaks corresponding to C_{21}, C_{23} and C_{25} aldehyde acetates in the high molecular weight region. This strongly indicates that the unsaturates are α,ω-diols, and by analogy and the NMR data above, it is fair to assume that the OH groups on the saturated diols are also α,ω. Furthermore, when the total sample of long chain diols was hydrogenated and run on capillary GC, the unsaturated species disappeared and the saturated peaks grew correspondingly larger.

The aldehyde acetates found were in the proportions $C_9 > C_7 > C_8$ in the low molecular weight region and $C_{23} > C_{25} > C_{21}$ in the high molecular weight region. This is exactly what one would expect if the pattern of unsaturation matches that of the ω-hydroxy fatty acids, i.e. there are more compounds with double bonds at $\omega 9$ than at $\omega 7$ which, in turn, exceeds the amount at $\omega 8$ (4). This strongly suggests that the 2 classes of lipid components have a biochemical precursor relationship, i.e., presumably the carbonyl groups of the ω-hydroxy fatty acids are reduced to form diols just as fatty acids are reduced to form fatty alcohols (9,10).

The data in Table 1 further support this conclusion. Here chain types of the α,ω-diols are compared with those of the ω-hydroxy fatty acids. Note that both lipid components have the same fatty chain types, i.e., straight even and odd (saturated and unsaturated) and iso plus anteiso (saturated and unsaturated). There are, however, more saturates in the α,ω-diols than in the ω-hydroxy fatty acids.

Human meibum also showed material with the same chromatographic behavior on SiO_2 and by GC as that of the steer α,ω-diols. Homologs are listed in Table 2. In as yet unpublished work we have found that the α,ω-diols and the ω-hydroxy fatty acids, both lipid components with 2 functional groups, are major components of 2 classes of lipid migrating by TLC on silicic acid in the diester region as well as the triester region (8). They also occur as polar monoesters. These novel compounds possibly play a role in the spreading of lipid in the precorneal tear film. Insight as to how this might occur must await the determination of the full structures of the lipids that contain these components.

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REFERENCES

1. Nicolaides, N., Kaitaranta, J.K., Rawdah, T.N., Macy, J.I., Boswell, F.M., and Smith, R.E. (1981) Invest. Ophthalmol. & Vis. Sci. 20, 522-536.
2. Musgrove, O.C., Stark, J., and Spring, F.S. (1952) J. Chem. Soc. 4, 4393-4397.
3. Kolattukudy, P.E. (1970) Biochem. Biophys. Res. Commun. 41, 299-305.
4. Nicolaides, N., Santos, E., and Papadakis, K. (1984) Lipids 19, 264-277.
5. Nicolaides, N., and Ruth, E.C. (1982) Current Eye Res. 2, 93-98.
6. Grob, K., and Grob Jr., K. (1978) J. Chrom., 311-320.
7. Stein, R.A., and Nicolaides, N. (1962) J. Lipid Res. 3, 476-478.
8. Nicolaides, N., Santos, E., and Smith, R.E. (1984) ARVO Abstract, Supp. to Invest. Ophthal and Vis. Sci., 25/4, p. 320.
9. Kolattukudy, P.E., ed. (1976) Chemistry and Biochemistry of Natural Waxes, Chapters 2,4,5, Elsevier, New York, NY.
10. Snyder, F., and Malone, B. (1970) Biochem. Biophys. Res. Commun. 41, 1382-1387.

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